STUDIES ON THE FATE OF AUTOXIDATION PRODUCTS OF LINOLEIC ACID ORALLY ADMINISTERED TO RAT(Dissertation_全文)

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ABBREVIATIONS

BHA	Butylated hydroxyanisole
BHT	Butylated hydroxy toluene
FA	Fatty acid
GC-MS	Gas chromatography-mass spectrometry
KX	Potassium halide
LA	Linoleic acid
LAHPO	Linoleic acid hydroperoxide
MA	Malonaldehyde
EtLA	Ethyl linoleate
MtLA	Methyl linoleate
9-ONA	9-oxononanoic acid
PP	Polar products
PV	Peroxide value
SP	Secondary autoxidation products
TBA	Thiobarbituric acid
TBARS	TBA reactive substances
TLC	Thin layer chromatography

I GENERAL INTRODUCTION

Polyunsaturated fats react easily with atmospheric oxygen to form their peroxides. In the presence of oxygen these peroxides are broken down to a complex mixture of various oxidized products, generally termed SP. These autoxidation products are a major cause of the deterioration of foodstuffs and also damage biologically significant compounds such as nucleic acid, amino acids and enzymes. Animals given oral doses of these products suffer from diarrhea, anorexia, growth suppression, hemorrhage and necrosis. Although the amounts of these autoxidation products may not always be high due to recent improvements in food technology, it is a fact that these toxicants occur in our daily food. On the other hand, lipid peroxides are also formed endogenously by active oxygen species. The endogenous lipid peroxidation products are not only associated closely with serious diseases [1-5] but are also a major factor of senescence [6-8] in animals. Then, from the standpoint of nutrition, the question whether or not these diseases are caused by the oral intake of autoxidation products, is an important problem urgently awaiting solution. As a first step, the fate in animal body of orally fed autoxidation products must be clarified.

In this study, LA labeled with ^{14}C was used and the autoxidation products were administered to growing male Wistar rats and their absorption and metabolism are discussed. In Chapter II, the autoxidation mechanism of LA is described. In Chapter III, the composition of the autoxidation products of LA are analyzed. In Chapter IV, the typical effects of these autoxidation products on proteins are described. Chapter V discusses the extent of incorporation into body when these autoxidation products were administered intragastrically to rats. Finally in Chapter VI, the detoxification and metabolism of SP to lipids in rat liver is described.

II PHYSICOCHEMICAL ASPECT IN AUTOXIDATION OF LINOLEIC ACID

II-1 INTRODUCTION

The autoxidation mechanism of olefins has been widely investigated since 1940. Several groups [9-12] clarified the formation of hydroperoxide as a first product in the autoxidation. Moreover, three kinds of processes were proposed to be involved in the initiation of autoxidation: (1) initiation by catalytic action of metal traces; (2) initiation by singlet oxygen which is formed from photosensitization reaction [13, 14] between triplet oxygen and a sensitizer such as chlorophyll; and (3) direct initiation by atmospheric oxygen. Hydroperoxides are formed by these reactions and a radical chain reaction is immediately triggered [15]. Initiation (1) was studied rather thoroughly [16-19] prior to 1971. Photosensitized oxidation (2) has recently been clarified mainly by two groups, Terao et al. [20-22] and Frankel et al. [23-26]. Although atmospheric oxygen seems to play an important role in both the initiation of autoxidation and the trigger reaction of chain propagation, initiation (3) has not yet been identified.

In this Chapter, the importance of atmospheric oxygen at the initiation is discussed. The initiation of LA autoxida-

tion was suppressed by some kinds of salts [27] and the decomposition of LAHPO by oxygen was kinetically measured [28].

II-2 MATERIALS AND METHODS

LA. [1-^{14}C]LA and [U-^{14}C]LA were supplied by New England Nuclear, Boston, USA. Cold LA (95% < pure) was purchased from Tokyo Kasei Kogyo Co. Ltd. [1-^{14}C]LA and [U-^{14}C]LA were diluted with cold LA to 583 kBq/mmol and 51.4 kBq/mmol (1 kilo Becquerel=27 nano Curie), respectively.

Preparation of LAHPO. LA was autoxidized at 37°C for 7 days in the dark under air. The autoxidized LA was diluted 3-fold with 2% methanolic benzene. The diluted solution was applied on a silica gel (Wakogel C-100) column (2.5x100 cm, the immobile phase was 20% methanolic benzene) [29, 30]. The elution was carried out at a rate of 50 ml/h, firstly with 1100 ml of 2% methanolic benzene, then with 600 ml of diethyl ether, and finally with 600 ml of methanol. The eluate was separated into 230 tubes (10 ml each) with a fraction collector. An aliquot of each fraction was subjected to TLC (Merck Kieselgel PF_{254} containing $CaSO_4$; 0.25x100x100 mm) for identification of the components. TLC

were developed with a solvent system of diethyl ether-hexane-acetic acid=60:40:1.5. Spots on the plate were detected by exposure to iodine vapor. The LAHPO fraction was further purified by prepared TLC (1x200x200 mm) twice. The band of LAHPO was detected under 254 nm light. Silica gel was scraped off the plate and placed in ethanol. The ethanol extract was obtained by centrifugation and the precipitated silica gel was again placed in ethanol for one more extraction. The ethanol solvent in the extract was evaporated and changed to hexane. The solution was centrifuged to completely remove the silica gel.

Characterization of autoxidation products. LAHPO was characterized by the PV method [31], UV spectrophotometry [32] and carboxyl titration with tetra-n-butylammonium hydroxide [33]. The purity of LAHPO was estimated to be more than 97%. The SP fraction was obtained from the diethyl ether eluate of the above silica gel column and was characterized by carbonyl analyses [34, 35]. The SP fraction was free of both LAHPO and LA as detected on TLC.

Polarographic analysis of oxygen absorption. Twenty milliliters of oxygen-saturated water containing $FeSO_4$ ($2x10^{-6}$ M) was put into a beaker and stirred continuously

with a magnetic stirrer under air [36]. The reaction mixture was covered over with liquid paraffin to prevent the mixture from being exposed to atmospheric oxygen before the insertion of a polarographic electrode (Beckman oxygen analyzer Model 39550). The beaker was placed in a water bath at 37°C and the electrode was stabilized. Then, 0.5 ml of LAHPO or SP methanol solution was injected into the water layer. The oxygen absorption was recorded with polarography (Yanaco Model P8). The oxygen-saturated water was prepared by bubbling air through water for 3 h and oxygen-free water was obtained by adding sodium sulfite.

Measurement of oxygen consumption with a respirometer. A Gilson differential respirometer Model GRP 14 and Warburg Manometer (Taiyo Kagakukogyo Co.) were used. In the Gilson Model, the main chamber contained 1.5 ml of sample emulsion and the side chamber contained 0.5 ml of reactant such as $FeSO_4$ solution. In the Warburg Manometer, the main chamber contained 0.9 ml of the reaction mixture (25 mM of LA or LAHPO, 0.1 M of buffer, 5% Tween 40 and various concentrations of salts) and the side chamber contained 0.1 ml of 0.1 mM $FeSO_4$ or 0.5 mM ethylene diaminetetraacetate, unless otherwise stated. After stabilization, the reactant was mixed with the sample emulsion and oxygen consumption was

read [37]. The reaction temperature was kept at 37°C and the chamber was continuously shaken.

Detection of autoxidation products on TLC. The reaction mixtures of LA and 1 M of KX (total volume of 200 ml) were incubated and continuously shaken at 37°C for 7 days. The reaction products were extracted several times with chloroform-methanol=1:1. The extracts were condensed and further purified several times on TLC as described above. The isolated LA and LAHPO were characterized by the weighing method and UV spectrometry, respectively. The spots of LAHPO and SP were detected by spraying 5% phenylhydrazine in 0.1 N HCl methanol solution [38]. With the phenylhydrazine reagent, an orange-red color was developed by LAHPO, and a yellow-red color was developed by SP.

Measurement of fluorescence. Absolute methanol was prepared using sodium metal and distillation. Twenty five millimoles of LA or MtLA was mixed with 250 mM of KI or 1 mM of I_2 in the absolute methanol solution. The solutions were excited with 315 nm wavelength and the fluorescence spectra were observed with a Hitachi Fluorescence Spectrophotometer 203 (mercury lamp) at near 410 nm.

Radioassay. The radioactivity was measured with a liquid scintillation spectrometer, Packard Model 2002 and Model 2425. The efficiency of each vial was calculated with a quenching calibration curve using an external standard, and then the radioactivity was expressed as Bq. An aqueous cocktail (consisting of 4 g of PPO, 0.1 g of POPOP and 75 g of naphthalene in 1 liter of toluene:dioxane:ethyl cellosolve=1:1:1 solution) and nonaqueous cocktail (6 g of PPO and 0.5 g of POPOP in 1 liter of toluene) were used. The radioactive substances adsorbed on TLC were mixed with Packard thixotropic gel powder (CAB-O-SIL) before the radioassay.

Procedure for removal of oxygen with Thumberg tube. LAHPO was put in a Thumberg tube and the catalyzer solution was placed in the side arm. The tube was cooled in liquid nitrogen and evacuated with a vacuum pump. Then, the tube was warmed at room temperature and argon was introduced. These procedures were repeated several times.

II-3 RESULTS

Oxygen absorption by LA, LAHPO and SP

LA, LAHPO and SP were emulsified into oxygen-saturated

water and ferrous sulfate was added as a catalyzer. LAHPO and SP consumed dissolved oxygen in the same manner LA (Fig. II-1). LAHPO, however, was more reactive to oxygen than LA.

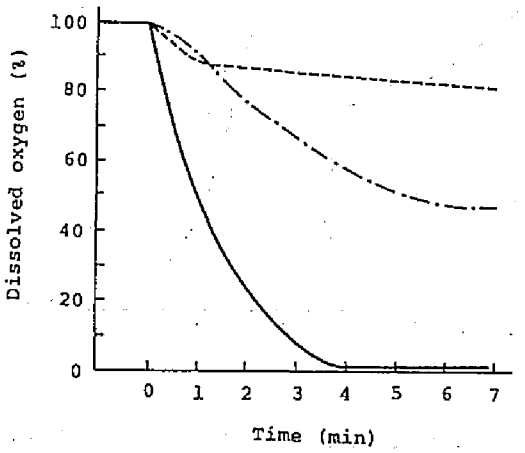


Fig. II-1. Dissolved Oxygen Consumption Measured with a Polarographic Oxygen Analyzer. The concentration of LA (— . — . —) and LAHPO (———) was 5 mM and that of SP (-----) was 10 mM as acetaldehyde (or 3 mM by PV).

The oxygen absorption rate of LA was the same as the rate at which atmospheric oxygen was dissolved into the solution 5 min earlier. Oxygen absorption by both LA and LAHPO was suppressed by the addition of radical scavengers such as uric acid [39] and BHA (Fig. II-2).

Total amounts of oxygen consumption by LA and LAHPO were measured with a respirometer (Table II-1). LA absorbed 2.36 moles of oxygen. LAHPO stoichiometrically absorbed 1 mole or more of oxygen in the presence and absence of a catalyzer.

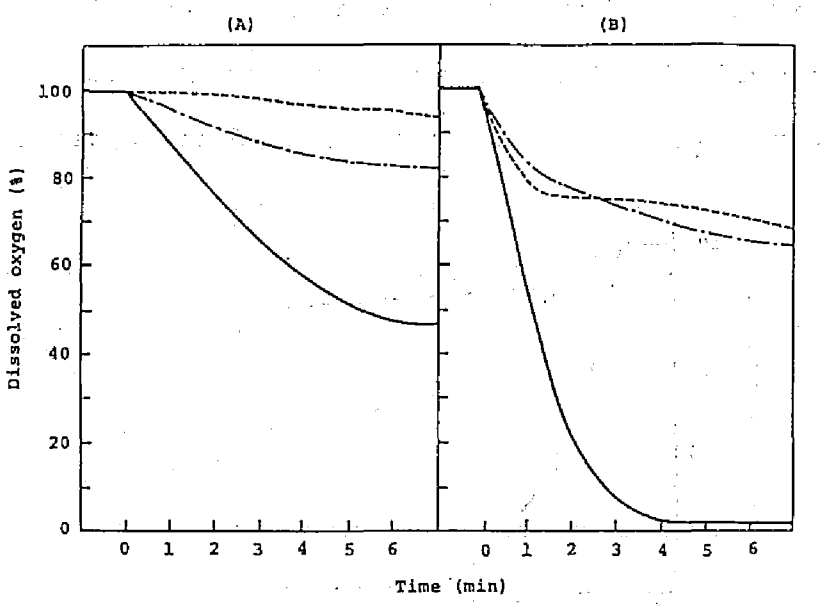


Fig. II-2. Effects of Antioxidants on the Oxygen Absorption of LA (A) and LAHPO (B). The reaction mixtures contained 0.5 mM of uric acid (— . — . —), 0.05 mM of BHA (-----), or nothing (———).

Table II-1

Total Oxygen Consumption of LA and LAHPO

Sample	addition of $FeSO_4$	for time* (h)	Consumed O_2 (μmol/μmol)
LA	20 mM	17	2.36
LAHPO	20-0.5 mM	18-23	1.25±0.07**
LAHPO	0	34	1.02

*The time it took for the reaction to reach the end.
**Mean±SE and n=4.

LAHPO was decomposed to SP when the oxygen absorption of LAHPO increased from 0.25 (mole/mole) to 1.0 (Fig. II-3). The spot of LAHPO was reduced and the number of spots of SP species increased on TLC. The weights per 51.3 kBq of [U-^{14}C]LAHPO and [U-^{14}C]SP were compared with that of [U-^{14}C]LA (Table II-2). The weight of LAHPO minus that of LA was

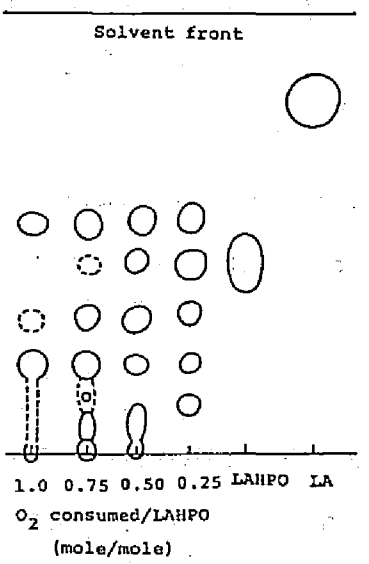


Fig.II-3. Decomposition of LAHPO with Oxygen Absorption. LAHPO was incubated in the respirometer. The reaction products were extracted and applied to TLC.

Table II-2

Weights of Autoxidation Products of [U-^{14}C]LA

	mg/51.3 kBq*
LA**	284±12***
LAHPO**	319±20
SP	370±17

*Because the specific activity of LA was 51.3 kBq/mmol.
**The purity of LA and LAHPO were 99 and 98%, respectively.
***Mean±SE (n=5).

319 - 280 = 39. Thus, LA absorbed 1.2 moles of oxygen (39÷ 32) and formed LAHPO. SP was produced by the reaction of LA with 2.8 moles of oxygen.

Antioxidative effects of salts

Antioxidative effects of KCl, NaCl and Na_2SO_4 on the oxygen absorption of LA were observed at pH 4.0 and 7.0 in the presence of $FeSO_4$ (Table II-3). The rate of oxygen absorption by LA was faster at pH 4.0 than at pH 7.0. Twenty five micromoles of LA finally took up 1300 µl of oxygen. KCl (at 1 M) was more antioxidative than NaCl or Na_2SO_4, while the ionic strength of Na_2SO_4 was twice that of KCl. Moreover, K^+ was more effective than Na^+ on the autoxidation of LA. The antioxidative effect of KCl was further observed in the micellar condition of LA (Table II-4). LA was emulsified into a solution containing 5% Tween

Table II-3

Effects of KCl, NaCl and Na_2SO_4 on the Autoxidation of LA

Incubation mixtures were composed of 25 µmoles of LA, 0.1 M of HCOOH-NaOH (pH 4.0) or KH_2PO_4-Na_2HPO_4 (pH 7.0) buffer, 0.1 mM of $FeSO_4$, and various concentrations of salts.

		Oxygen absorption (µl) for 40 h				
Salt at	(M)	0	0.01	0.1	1	2.5
	KCl	1175	968	376	343	441
at pH 4	NaCl	1082	948	491	415	498
	Na_2SO_4	1072	1078	1097	644	-
	KCl	445	288	234	101	194
at pH 7	NaCl	441	754	431	124	191
	Na_2SO_4	410	378	388	136	-

Table II-4

Effect of KCl on the Oxygen Absorption of LA in Micellar and Non-micellar Systems

The incubation mixtures consisted of 25 μmoles of LA or EtLA, 0.1 M of buffer (pH 7.0), 0.1 mM of $FeSO_4$, with 5% of Tween 40 or without, and KCl.

		Oxygen absorption (μl) after 40 h		
		KCl (M)		
Sample	Tween	0	0.1	1
LA	without	601	511	397
LA	with	445	236	102
EtLA	with	115	21	22

40 [40]. The autoxidation rate of LA in the system without Tween 40 (micellar) was faster than that in the system with Tween 40 (non-micellar). The antioxidative effect of KCl was clearly evident in the non-micellar system. The effect of KCl on the autoxidation of EtLA was also antioxidative and thus had no correlation with the micelle of LA. Fig. II-4 shows the effects of salts on the oxygen absorption of LA in the absence of a catalyzer. Na_2SO_4, KI and KOCN were antioxidative, and KF, KBr and $Na_2S_2O_3$ were prooxidative. KCl also had an antioxidative effect at 1 M on the oxygen absorption of LAHPO (Fig. II-5). The salts (at 1 M) also

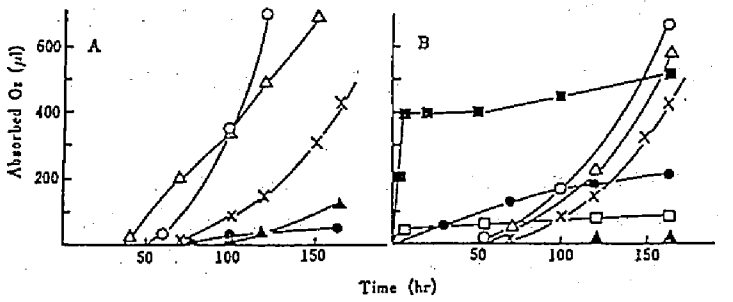


Fig. II-4. Effects of Salts on the Oxygen Absorption of LA. The incubation mixtures consisted of 25 mM of LA, 0.1 M of buffer, 0.5 mM of ethylenediamine tetraacetate, 5% of Tween 40 and 1 M of salts: —○— , KF; —△— , KBr; —●— , KI; —▲— , Na_2SO_4; —×— , nothing; in A; and —○— , KNO_3; —△— , 0.2 M of $KClO_4$; —□— , KSCN; —●— , KCN; —▲— , KOCN; —■— , $Na_2S_2O_3$; —×— , nothing; in B.

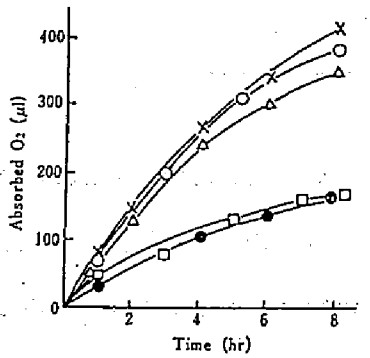


Fig. II-5. Effects of KCl on the Oxygen Absorption of LAHPO. The oxygen consumption by 20 umoles of LAHPO was measured with the respirometer in solution of: —●— , 2.5 M; —□— , 1 M; —△— , 0.1 M; —○— , 0.025 M and —×—, 0 M of KCl. The incubation mixture was the same as Table II-3.

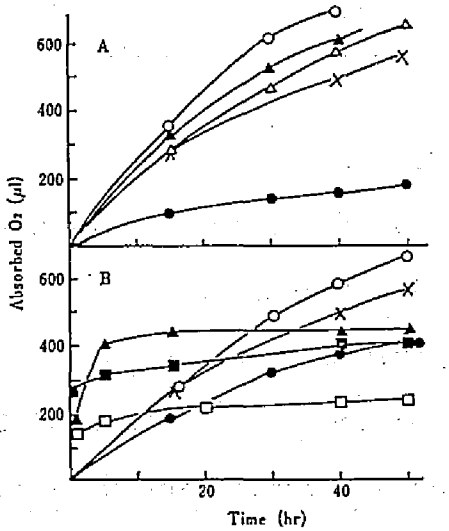


Fig. II-6. Effects of Salts on the Oxygen Absorption of LAHPO. The incubation mixtures contained 29 umoles of LAHPO and the other components were the same as Fig. II-4.

exhibited antioxidative effects on LAHPO oxidation (Fig. II-6). These effects of KX followed the increasing order KF < nothing < KCl < KI. Thus, KI was most antioxidative.

Amount of oxygen consumed by LA in salt solution

Total amounts of the oxygen consumed by LA and LAHPO were determined in the KX solution containing an excess of $FeSO_4$

Table II-5

Total Amounts of Oxygen Consumed by LA and LAHPO in KX solution

LA was incubated with the respirometer in KX solutions containing 5 mM of $FeSO_4$ and LAHPO was incubated in 0.5 mM of $FeSO_4$. Hydroxyl derivatives (HD) of LAHPO were prepared by reduction with KI. LAHPO (27 μmoles) was boiled with 160 μmoles of KI under nitrogen gas for 10 min. The products were extracted, washed several times with $Na_2S_2O_3$ and centrifuged.

Sample (μmoles) in	Total absorbed amount of oxygen (μl) KI	KBr	KCl	KF	None	Incubation time (h)
25 of LA	ND*	1113	1139	1479	1271	77
re-supply**	ND	1191	1220	1502	1428	100
27 of LAHPO	104	558	514	870	790	100
re-supply	119	582	545	870	800	125
27 of HD	-	-	-	-	585	50

*The oxygen absorption was not detected.

**The oxygen absorption was determined by one more addition of $FeSO_4$.

(Table II-5). Oxygen absorption of samples was measured and after one more supply of $FeSO_4$ the total oxygen consumption was determined. LA did not absorb oxygen in KI solution. Although LAHPO finally consumed 800 μl of oxygen in a salt-free system, LAHPO consumed 119 μl of oxygen in KI solution. LAHPO might be reduced by KI to hydroxyl derivatives and KI might be converted to I_2 [41]. The hydroxyl derivatives, however, consumed 585 μl of oxygen. The total amount consumed was (585 μl plus 119 μl) close to the amount consumed by LAHPO (800 μl). The effect of I_2 was then examined. Fig. II-7 shows the prooxidative effect of I_2 (at 1-25 mM) on the oxygen absorption of LA.

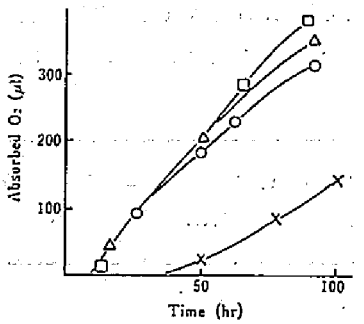


Fig. II-7. The effect of I_2 on the Oxygen Absorption of LA. The incubation mixtures consisted of the same components as in Fig. II-4 except —○— , 25 mM; —△— , 10 mM; —□— , 1 mM and —×—, 0 mM of I_2.

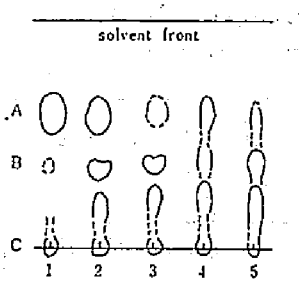


Fig. II-8. TLC of the Autoxidation Products of LA in KX Solutions. Nos. 1 to 5 were the extracts of autoxidation products of LA in KI, KBr, KCl, KF and no salt solutions, respectively.

The reaction products of LA in the KX solution were extracted and applied to TLC. Three major spots (A), (B) and (C) were observed (Fig. II-8) and their R_f values agreed with those of authentic LA, LAHPO and SP species, respectively. LA remained unchanged in KI solution. The spots of LAHPO and SP species were enlarged in the order of KBr < KCl < KF solutions. Then, the spots (A) and (B) were isolated and submitted to one more determination of oxygen consumption (Table II-6). Spot (A) consumed 1.7 μmoles/mol of oxygen, 70% of the final amount consumed by LA. Spot (B) absorbed 50% of the final amount consumed by LAHPO. MtLA was incubated in KX solutions and the reaction products were extracted as in the case of LA. Spot (A) was isolated on TLC and analyzed with GC-MS (Shimazu LKB 9000) using SE-52 (3%) on Chromosolve W under the same analytical conditions as reported by Terao et al.

Table II-6

Amounts of Absorbed Oxygen by Spots (A) and (B)

Spots (A) and (B) were isolated from TLC as shown in Fig. II-8. Total amounts of absorbed oxygen were determined in duplicate with the respirometer. The system containing 5 mM of $FeSO_4$ was incubated for 70 h.

Sample	Absorbed oxygen (mol/mol)
Spot (A)	1.72
Authentic LA	2.32
Spot (B)	0.76
Authentic LAHPO	1.41

[42]. Spot (A) gave the same mass fragment ions as that of authentic MtLA [43]. Therefore, it was concluded that LA at the initiation process of autoxidation was reversibly stabilized by KI but not irreversibly by the addition of I_2.

Complex formation of LA with salts

The possibility of a complex formation between LA and halide ions was examined. When spot (A) was dissolved in methanol and excited, fluorescent light was observed at 380 nm (Fig. II-9). The fluorescent light was also measured using pure LA and MtLA. An absolute methanol mixture of LA with KI was excited at 315 nm and fluorescence was observed at 412 nm 30 sec earlier (Fig. II-10). The fluorescence shifted to a long wavelength (at 425 nm) after 5 min following the development of a yellow color of KI. Fluorescence in the mixture of LA with I_2 was also observed and the light peak (410 nm) was at a different position from the peak of the mixture with KI. Fig. II-11

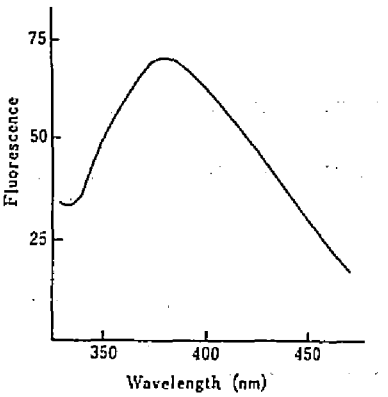


Fig. II-9. Fluorescence of Spot (A). Spot (A) (90 mg) on TLC in Fig. II-8 was dissolved in 2 ml of absolute methanol and excited at 315 nm.

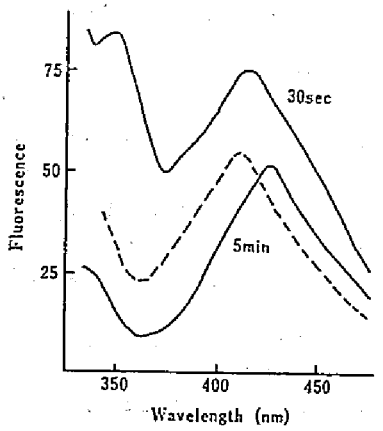


Fig. II-10. Fluorescence Spectra of LA with KI (————) or I_2 (-----). LA was mixed with KI and measured after 0.5 and 5 min.

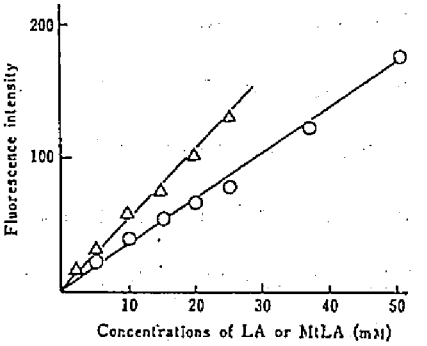


Fig. II-11. Dependence of Fluorescence Intensity on the Concentrations of LA (o—o) and MtLA (△—△). The fluorescence was observed at 412 nm after 0.5 min of the mixing.

shows that intensities of the fluorescence depended on the concentration of LA or MtLA. Therefore, the fluorescence of the LA-KI mixture may be ascribed to the complex of LA and iodide ion.

Kinetic analyses of oxygen absorption by LAHPO

Rate constants of oxygen absorption by LAHPO were determined by both the polarographic and respirometric methods and expressed by the following formulas. The rate of reaction can be expressed as the decreasing rate of oxygen concentration $[O_2]$ at any time. If the chosen substance is a reactant, the rate $v = -d[O_2]/dt$. This reaction involves

two reactants, i.e., LAHPO and oxygen. Therefore, the rate can be expressed in relation to the concentration of reactants by the equation $-d[O_2]/dt = k[LAHPO]^m[O_2]^n$, where k is the rate constant and m and n are orders of the reaction. The reaction is determined to be the mth order with respect to LAHPO, and the nth order with respect to oxygen. If one reactant is in excess of the other, the rate equation can be expressed in simply. For polarographic determination, 4.5 μmoles of oxygen were dissolved in the reaction mixture. When LAHPO is in large excess of oxygen, such as in the case of Fig. II-12 (A), the other reactant $[O_2]$ will disappear with time. The decrease of $[O_2]$ is equal to $([O_2]_0 - [O_2]_t)$. Then the rate equation of the differential form is $-d[O_2]/dt = k'([O_2]_0 - [O_2]_t)^n$, where $[O_2]_0$ is the concentration of dissolved oxygen in the initial stage, and $[O_2]_t$ is the concentration in time t. Here, when this equation integrates with n=1 (first order), the integration coefficient is denoted by the initial concentration of oxygen at time t = 0, that is, $\ln\{[O_2]_0/([O_2]_0 - [O_2]_t)\} = k't$. The applicability of this equation can be tested by the determination of k't at various time intervals. Plots of $\log[O_2]_0/([O_2]_0 - [O_2]_t)$ versus time should give a straight line. Fig. II-12 (A) shows the k'. Thus, the absorption of oxygen by LAHPO followed the first-order reaction with

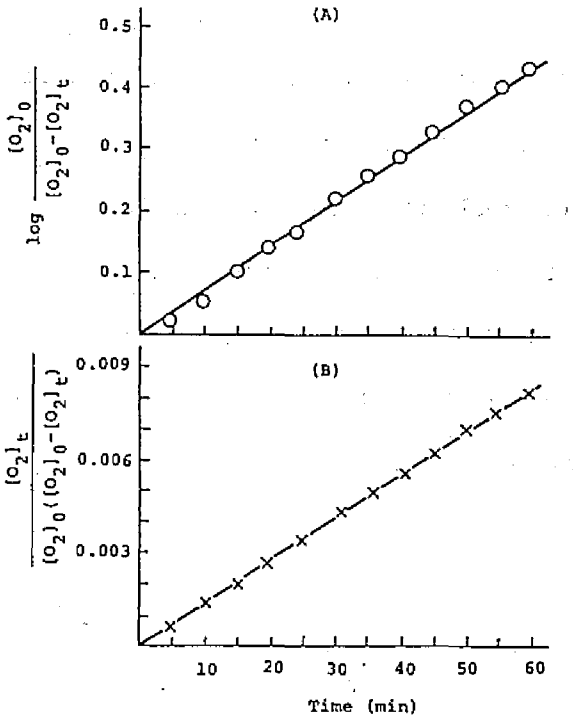


Fig. II-12. The Kinetic Curves of Oxygen Absorption by LAHPO from Polarographic Determination. When LAHPO (100 μmoles) was in excess of dissolved oxygen (4.5 μmoles) (A); ——o——, the rate constant was 0.469 min^{-1}. When LAHPO and dissolved oxygen were in an equimolar proportion system (4.5 μmoles each) (B); ——x——, the constant was 0.00838 min^{-1}.

respect to oxygen.

If 1 mole of oxygen is consumed by 1 mole of LAHPO, the decrease of dissolved oxygen equals the decrease of LAHPO. When these reactants are set in equimolar proportion, the reaction follows the second-order. The applicability of this equation was tested and Fig. II-12 (B) shows the

straight line of k when $[O_2]_0/[O_2]_0([O_2]_0 - [O_2]_t)$ versus time was plotted.

Moreover, the kinetic orders were observed with a respirometer, in a system containing an excess of oxygen. The equation was calculated with m = 1 following the same method used for polarographic determination. The plots of $\log[O_2]_0/([O_2]_0 - [O_2]_t)$ should give a straight line. The constant k was determined with various concentrations of the catalyzer. Fig. II-13 shows that the reaction followed the

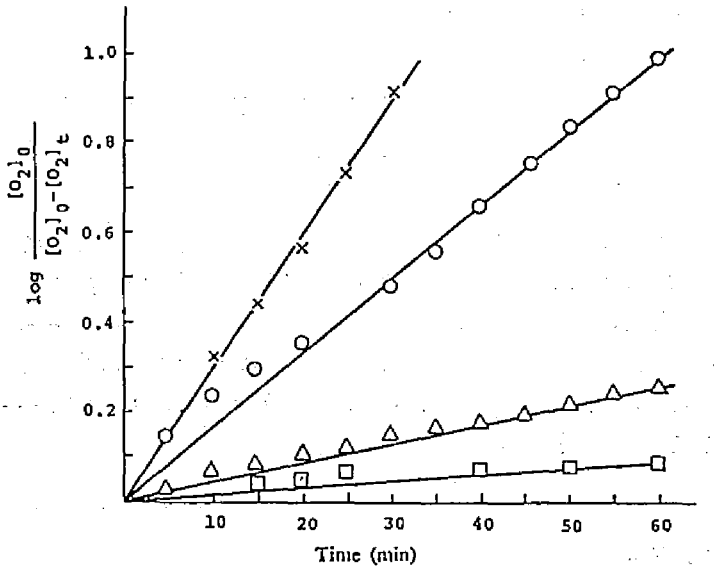


Fig. II-13. The Kinetic Curves of Oxygen Absorption from LAHPO in the Respirometric Determination. The concentration of LAHPO was 10.7 μmoles and the gas phase volume of the respirometric chambers was 25 ml. The concentration of $FeSO_4$ which was added to the reaction mixture was varied as follows: ——x——, 40 mM (the rate constant was 0.0295 min^{-1}); ——o——, 10 mM (0.0153); ——Δ——, 1 mM (0.00390); ——□——, 0 mM (0.000991).

first-order with respect to LAHPO.

The half-life method was used when the catalyzer was kept constant and the question whether or not other steps were involved in the absorption reaction was clarified (Fig. II-14). The half-life is the time taken for one-half of the original concentration of a substance (LAHPO) to disappear and is closely dependent on the rate constant. Although the concentration of LAHPO in the initial stage was changed to 10.7 μmoles, 8.6 (4/5), 6.4 (3/5), and 4.3 (2/5), the rate constants remained unchanged, 0.0246±0.0014 (mean±SE).

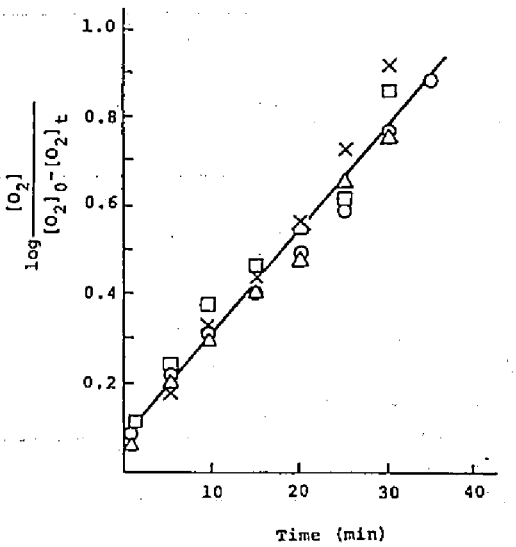


Fig. II-14. Determination of Rate Coefficient with Half-life Method. In the respirometer, the concentration of $FeSO_4$ was constant (0.35 mM) and LAHPO concentration was changed as follows: ——x——, 10.7 μmoles (the rate coefficient was 0.0287 min^{-1}); ——o——, 8.6 μmoles (0.0225); ——□——, 6.4 μmoles (0.0232); ——△——, 4.3 μmoles (0.0240).

Thus, the reaction of LAHPO with oxygen seemed to be an elementary equation.

Reactivity of oxygen to LAHPO

[1-^{14}C]LAHPO was incubated for 1 h in a Thumberg tube in the absence of oxygen under the same conditions as in the

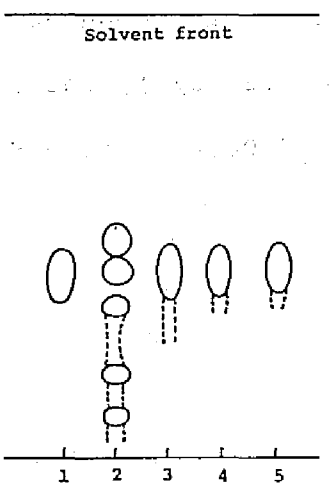


Fig. II-15. Incubation of [1-^{14}C]LAHPO in the Absence of Oxygen. LAHPO was incubated in a Thumberg tube for 1 h and cooled by liquid nitrogen. Ether (2 ml) was injected into the tube under argon. The tube was shaken after exchanging air with argon. The ether extracts were quantitatively applied to TLC under argon. Chromatogram Nos. 1, authentic LAHPO; 2, the incubated LAHPO (6.5 μmoles) at 37 °C for 1 h under air; 3, 4 and 5, the incubated LAHPO (6.5, 13.0 and 15.2 μmoles) without oxygen.

Table II-7

Recovery % of [1-^{14}C]LAHPO after Incubation under Oxygen-free System

incubation under	time (h)	recovery % of LAHPO[a]
O_2-free	0	96.4[b]
O_2	1	22.9[b]
O_2-free	1	83.1±4.3[c]

a, Radioactive 6.5-15.2 μmoles of LAHPO were used. The purity of LAHPO was > 91.6% on the TLC and these recovery % were divided by 0.916.
b, Means of two experiments.
c, Mean±SE with n=6.

case of Fig. II-14. LAHPO remained unchanged on TLC (Fig. II-15). The recovery % of LAHPO was determined radiochemically (Table II-7). While 80% of LAHPO was decomposed under air for 1 h, LAHPO was stable without oxygen even in the presence of both a catalyzer and heat. Therefore, it was concluded that oxygen played an important role in the decomposition of LAHPO.

II-4 DISCUSSION

Recently, the autoxidation mechanism of FA has received much attention [44-48]. However, not much has been reported on the initiation step. There has been discussion on whether the autoxidation of polyunsaturated FA is initiated by an abstraction of hydrogen atom from active methylene [49] or not [50]. The data in this Chapter show that oxygen triggered both the autoxidation of LA and decomposition of LAHPO. LA was changed to LAHPO by 1 mole of oxygen and LAHPO was decomposed to SP by more than 1 mole of oxygen (Fig. II-1~3, Table II-1 and 2).

The former reaction was suppressed by some kinds of salts. The effects of the salts were not correlated with their ionic strength (Table II-3). The effects were stronger in a micellar form of FA than under the permissible

condition of a detergent (Table II-4). The antioxidative effects of KX were in the increasing order $F^- < Br^- < Cl^- < I^-$ (Fig. II-4~6) and were not caused by the addition of halide ions to double bonds (Table II-5, Fig. II-7 and 8). This order paralleled the increase of ion radiuses of these ions. It is well known that a large ion such as iodine is a π-electron receptor [51, 52]. The complex between KI and LA was detected by fluorescence (Fig. II-10 and 11). Campbell et al. [53] also suggested that the initiation of autoxidation of LA involves a process such as the formation of a complex between LA and atmospheric oxygen. It was concluded that the salts formed a reversible complex with LA and contributed antioxidatively (Table II-6 and Fig. II-9).

The latter reaction was kinetically observed in the steady state. LAHPO was more sensitive to oxygen than LA and SP. This reaction followed the first-order (Fig. II-12 and 13). Stoichiometrically, 1 mole of LAHPO reacted with 1 mole of oxygen (Table II-1 and 2). This reaction was a bimolecular one between LAHPO and oxygen, and did not include complex steps such as hydrogen abstraction (Fig. II-14). LAHPO was not polymerized nor decomposed under the oxygen-free condition (Fig. II-15 and Table 7). Gardner et al. [54] also reported that LAHPO did not generate any radicals under nitrogen atmosphere. Therefore, it was concluded that

the oxidative decomposition of LAHPO was initiated by the direct attack of oxygen.

On the basis of these results, the role of oxygen in the autoxidation process of LA is schematically illustrated in Fig. II-16. Polymers (1) are always produced in a termination reaction [55-57]. HOOLOO· (2) is a dihydroperoxide and may be formed as a reaction intermediate. The formation of this intermediate was supposed by the present data of kinetic observations and also supported by the findings of Terao and

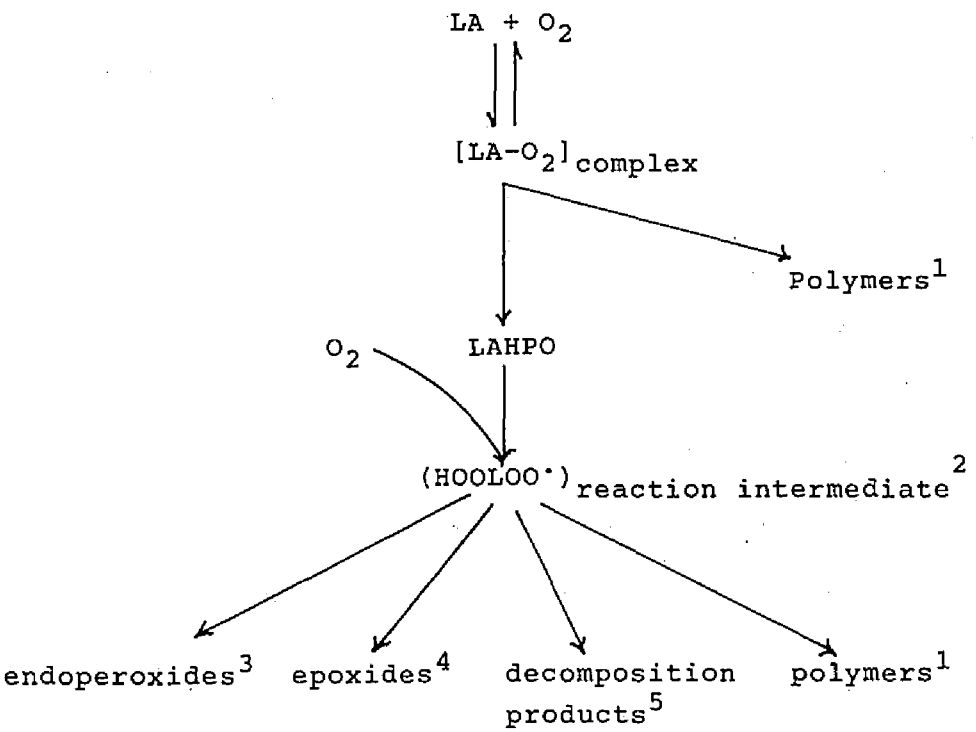


Fig. II-16. Proposal of the Autoxidation Mechanism of LA.

Matsushita [58], and Coxon et al. [59]. Endoperoxides (3) are easily formed by cyclization of one peroxyl group of dihydroperoxide and has been identified [60-64]. Epoxides (4) are also formed by cyclization [65-67]. Dihydroperoxide may be unstable and is easily decomposed (5) to various products [68-70]. These decomposed products are described in the next Chapter.

III ANALYTICAL OBSERVATION OF AUTOXIDATION PRODUCTS

III-1 INTRODUCTION

LA is autoxidized to LAHPO as a first product. LAHPO is broken down under atmospheric oxygen and gives SP, as described in Chapter II. SP is a complex mixture of many kinds of further oxidized products such as polymers, carboxylic acids [71], dicarboxylic acids [72], epoxides [73, 74], endoperoxides, aldehyde compounds [75, 76], peroxy alkenals [77], MA [78], and so forth. The toxic effects of autoxidation products appear to be due to a sum of these various compounds [79]. Therefore, the determination of the complete composition of autoxidation products is an important problem from the standpoint of food chemistry.

In this Chapter, this problem was clarified using LA uniformly labeled with ^{14}C, on the assumption that the amount of ^{14}C in the system does not change throughout the autoxidation of LA. Furthermore, an attempt was made to obtain information on TBARS contained in the autoxidation products [80].

III-2 MATERIALS AND METHODS

Autoxidation of [U-^{14}C]LA. Radioactive LA was diluted to the specific activity of 51.4 kBq/mmol and autoxidized at 37°C in the dark under three conditions referred to as "Closed," "Open" and "Rotating." Under the "Closed" condition 25 g of LA was placed in a small petri dish (ø 75 mm) and kept covered. Under the "Open" condition, 5 g of LA was placed in a large petri dish (ø 120 mm) without a cover and stirred twice a day. Under the "Rotating" condition, 5 g of LA was put in a 200 ml volume egg-shaped flask, which was continuously rotated to keep LA in thin film condition under air.

Analyses of autoxidation products. Yield % of the autoxidation products was measured by radioassay as described in Chapter II. The concentration levels of -COOH, -OOH and -CHO groups in the autoxidation products were determined by titration with tetra-n-butylammonium hydroxide [33], PV [31] and carbonyl analyses [34, 35], respectively.

TBA test. TBA test was carried out following one of the methods proposed by Matsushita [81]. The reaction mixture was composed of a certain volume of sample methanol solution, 2 ml of 0.5% TBA reagent, 0.1 ml of BHT (2.5×10^{-2} M in ethanol) and 3 ml of 50% acetic acid. This reaction mixture

was saturated with nitrogen and heated for 15 min in a boiling water bath. The colored water layer was washed with 4 ml of an acetic acid-chloroform=2:1 mixture and the optical density was measured at 532 nm.

Sephadex LH-20 gel filtration. Sephadex LH-20 was preswollen in ethanol and packed into a 2.5x78 cm column. One milliliter of a sample ethanol solution containing 380 mg and 52.7 kBq of SP was applied on the column, and eluted with ethanol at the rate of 30 ml/h. Fractions (5 ml each) were collected with a fraction collector.

Reduction of SP with $NaBH_4$. Radioactive SP (821 mg) ethanol solution was added to 50 ml of water at pH 9.5 and then 150 mg of $NaBH_4$ was added to the solution [82]. This mixture was allowed to stand at 37°C for 23 h. The reduced products were extracted 5 times with diethyl ether.

Methyl esterification. SP was methylated in diethyl ether with diazomethane gas. The gas was generated from p-toluenesulfonyl-N-methyl-N-nitrosoamide in diethylene glycol monoethyl ether with potassium hydroxide [83].

GC-MS analysis. The methylated SP was applied to a

Hitachi GC-MS, Model 6MG, with a 2 m column of Silicon Gum SE-52 (3%) on Chromosolve W, 60-80 mesh, or a 1 m column of Silicon OV-1 (2%) on gas chrom Q, 60-80 mesh. The analytical conditions were elevation of the temperature from 80°C to 250°C at 20°C/min; flow pressure of helium gas of 1 kg; ionic voltage of 20 eV; and a sample temperature of 100°C and a chamber temperature of 200°C.

Quantitative GC analysis. Some components of SP were quantitatively determined with a Shimazu GC, Model 6AM with a Silicon Gum SE-52 column, using an FID detector. The operational conditions were an increase in temperature from 80°C to 250°C at 5°C/min; a flow rate of 40 ml/min of nitrogen gas; and injection and detection temperatures of 270°C. Compounds on the chromatogram were quantified from their HW values according to the method of A.O.A.C. [84].

Sephadex G-10 chromatography for detection of MA. An attempt was made to detect MA in SP by the method of Kwon and Olcott [85]. SP (290 mg and 40 kBq) was added to 1 ml of Tris buffer and stirred sufficiently, and centrifuged. This extraction procedure was repeated 3 times. The extract, which was the water-soluble part of SP, was applied on a 2.5x92 cm column of Sephadex G-10. Elution was carried out

with 0.05 M Tris buffer containing 0.1 M of sodium chloride (pH 7.4) at a flow rate of 50 ml/h. The void volume of the column determined with blue dextran 2000 was 165 ml.

III-3 RESULTS

Autoxidation of LA under three conditions

Autoxidation of LA was observed under three conditions which varied the contact with atmospheric oxygen (Table III-1). In the case of "Closed" autoxidation, the surface area of LA was limited to the atmosphere. LA weight increased

Table III-1

Increasing Ratio of Weight of LA during the Autoxidation

LA was autoxidized under three conditions and the increasing weights at intervals were each compared with the initial weights. The SP fraction was obtained as illustrated in Fig. III-1 and the specific activity of SP was compared with that of LA.

Autoxidation	Period (days)						SP fraction
	0	4	7	14	21	28	
Closed	1.00	1.02	1.03	1.06	1.08	1.08	-
Open	1.00	1.08	1.11	1.15	1.17	1.19	1.27±0.01*
Rotating	1.00	1.10	1.14	1.17	1.17	-	1.29±0.02**

*Mean value±SE (n=3) of SP after 21 days autoxidation.
**(n=5) of SP after 7 days autoxidation.

and reached a maximum after 21 days, 1.08-fold. Under the "Open" condition, LA was more in contact with oxygen than under the "Closed". The maximum weight was 1.17-fold around 21 days. Under the "Rotating" condition, oxygen was continually supplied to LA and the maximum weight was 1.17-fold after 14 days. The autoxidation rate under "Rotating" was the most rapid of the three conditions.

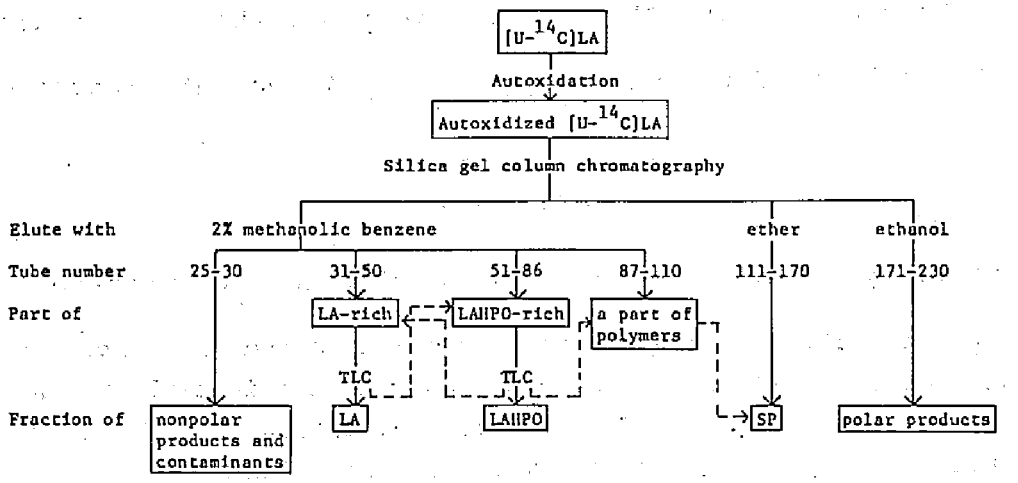


Fig. III-1. Separation Procedure for Autoxidation Products of [U-^{14}C]LA. The autoxidized LA was separated into five fractions by silica gel column chromatography as described in Chapter II. The fraction of tube Nos. 25-30 corresponded to a mixture of nonpolar products [86]. Nos. 31-50 and 51-86 belonged to the LA-rich and LAHPO-rich fractions, respectively. Nos. 87-110 corresponded to a part of the polymeric products [87]. A mixture of Nos. 87-110 and the ether eluate [29] was called SP. The methanol washing fraction was considered a mixture of PP. Both the LA- and LAHPO-rich fractions were purified by TLC and the other bands on TLC were returned to their own positions, as shown by broken arrows.

The autoxidation products of [U-^{14}C]LA were chromatographically separated. The schematic process is shown in Fig. III-1. A comparison was made of the composition of autoxidation products between "Rotating" after 7 days and "Open" after 21 days (Table III-2). LA absorbed an amount of oxygen which equaled 14% of the weight of intact LA in the former autoxidation and absorbed 17% in the latter. The "Rotating" autoxidized LA contained a large amount of LAHPO, 18%, while the "Closed" autoxidized LA consisted of a large part of SP, 54%. In the far right column of Table III-1, the ratio of the increase in the SP weight after "Rotating" for 7 days to the LA weight, 1.29±0.02-fold, was the same as that after "Open" for 21 days, 1.27±0.01. Therefore, the

Table III-2

Yield Percents of Autoxidation Products from [U-^{14}C]LA

Autoxidation*	Products				
	Nonpolar products	LA	LAHPO	SP	PP
Open** for 21 days	<0.01	22.4±3.5	7.8±0.8	54.3±4.7	15.4±3.2
Rotating*** for 7 days	<0.1	44.8±4.9	17.6±2.9	34.6±1.8	2.9±0.8

*The radioactivity of LA was 918 kBq.

**Mean±SE (n=3).

***Mean±SE (n=5).

yield percent of SP changed with the amount of absorbed oxygen, but the character did not.

Changes in function groups from the autoxidation process

The autoxidized LA after "Rotating" for 7 days was analyzed. Table III-3 shows the concentrations of three functional groups in the LA, LAHPO and SP fractions. The purities of LA and LAHPO were 99% and 98%, which were

Table III-3

Changes in Functional Groups

from the Autoxidation Process of LA

The concentrations of functional groups were converted to a standard of 51.3 kBq of radioactivity, because the purified LA from the autoxidation products had a specific activity of 51.3 kBq/mmol.

Functional group	Concentration (μeq)* in		
	LA	LAHPO	SP
-COOH	1000±14	968±47	1082±30**
-OOH	ND***	950±29	251±53
-CHO	-	-	470±25

*Mean±SE with n=5.

**A statistic significantly different from the value of LA ($p < 0.05$).

***Not detectable.

determined from their weights (Table II-2) and concentrations of the functional groups. Aldehyde groups were also detected in the SP fraction by a distillation method [34]. Most of the carbonyl groups in the SP fraction was aldehydes. The concentration of the -COOH group in SP was a little more than that in LA. The -OOH group in SP decreased to one-fourth of that in LAHPO and the -CHO group appeared. PP (Table III-2) consisted mainly of carboxylic acids.

Components in SP

Fig. III-2 shows that SP could be separated on Sephadex LH-20 radiochemically into two parts at 260 ml of elution volume (shown with a vertical line A). The part of molecular-weight higher than that of LA occupied 62% of the applied radioactivity and the part lower occupied 38%. The part of higher molecular-weight was further separated into two parts at 230 ml of elution volume (vertical line B). In the former part (36%), some peaks which appeared seemed to be polymers. Substances in the latter part were UV inactive and peroxide reaction positive. Dihydroxystearate derivatives were detected by MS from their hydrogenated silylated materials. Therefore, the latter part was considered to be a mixture of epoxyhydroperoxides- or endoperoxides-rich [25].

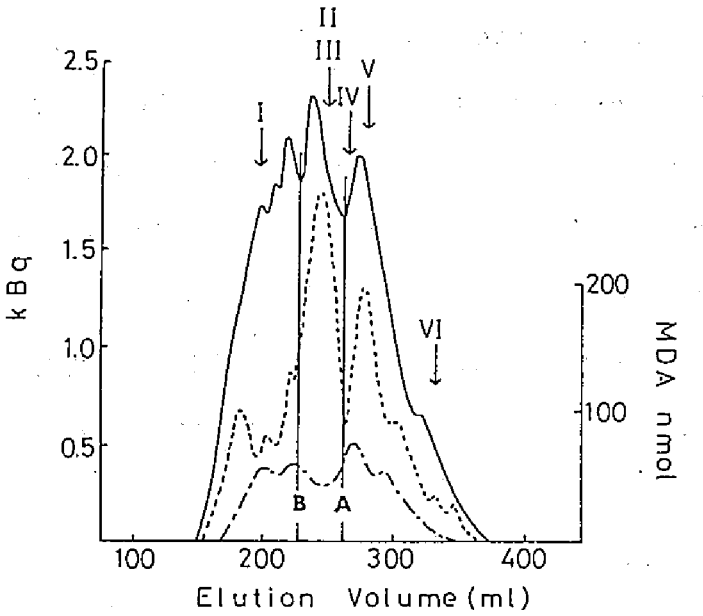


Fig. III-2. Sephadex LH-20 Gel Filtration of SP. Two milliliters each of the fractions were assayed for radioactivity (———) and TBARS (-----). Six authentic samples were eluted and detected by UV absorption. The elution positions are indicated by arrows numbered I; triolein (whose molecular weight was 885), II; behenic acid (340), III; LAHPO (312), IV; LA (280), V; lauric acid (200) and VI; hexanal (100). The elution pattern of the reduced SP was expressed at half scale of radioactivity (—·—·—).

SP was reduced and 320 mg (42.7 kBq) of that was applied on the gel. Radioactivity of the higher molecular weight part decreased to 57%. Unstable components such as epoxides and endoperoxides might have decomposed.

The lower molecular-weight part seemed to be a mixture of decomposed products. An attempt was then made to identify these components by GC-MS. SP was modified by methyl

esterification and 0.178 mg of that was subjected to GC using two kinds of columns. The chromatogram with SE-52 is shown in Fig. III-3 and is similar to the observation made with OV-1. Ten major peaks on GC were numbered and subjected to MS. Mass fragmentations of peak Nos. 1, 5, 9 and 10 are seen in Fig. III-4. Peak No. 1 was identified as hexanal, because it gave the same ions as those of an authentic sample, i.e., a weak molecular ion; m/z: 100 and some fragment ions; m/z: 71 (M^+-CHO), 58 ($CH_2CH_2CHOH^+$) and 43 ($CH_3CH_2CH_2^+$). Peak Nos. 2-4 showed typical fragmentation of fatty acid methyl-

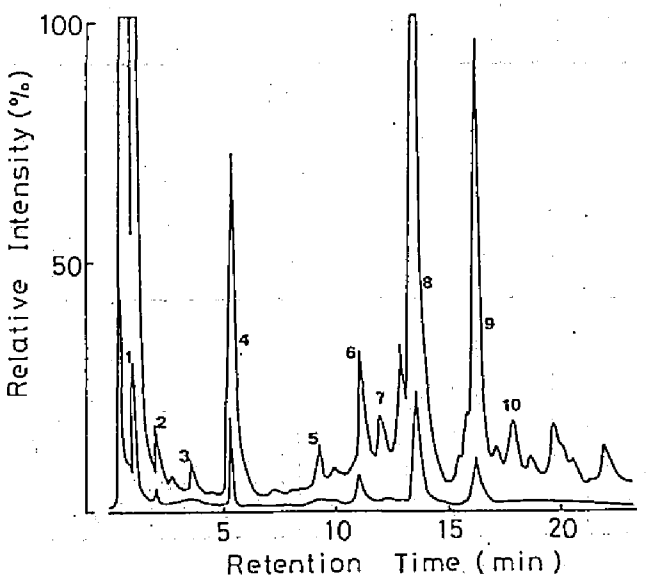


Fig. III-3. Gas Chromatogram of Methylated SP. The methylated SP was subjected to GC with a column of Silicon Gum SE-52. The major peaks were numbered 1 to 10 in order of observation. The lower line was recorded at one-sixteenth the level of the upper line.

esters, ($M^+ - CH_3O$) and ($CH_2CH_2COOCH_3^+$). Peak Nos. 5 and 7 had major ions of m/z: 182 (M^+), 125 ($M^+ - CH_2CH_2CH_2CH_3$) and 98 ($CH_2=C=CHCOOCH_3$) or 109 ($M^+ - CH_2COOCH_3$). These compounds appeared to be isomers of methyl decadienoate according to

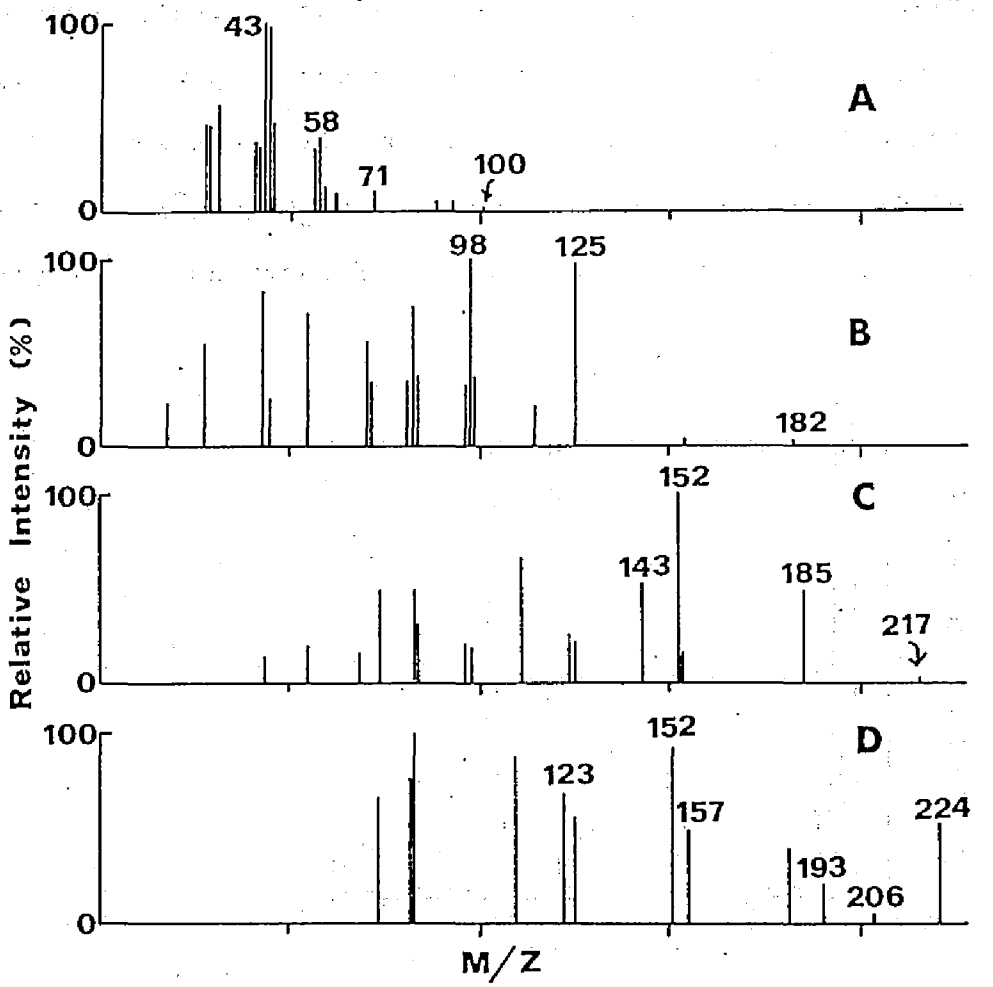


Fig. III-4. Mass Spectra of Low Molecular-Weight Components of SP. Ten peaks on GC (Fig. III-3) were subjected to MS analysis. The MS fragmentations of peak Nos. 1 (Fig. A), 5 (B), 9 (C) and 10 (D) are illustrated.

the analysis of Matthews et al. [88]. Peak No. 6 gave major ions of m/z: 172 (M^+), 154 ($M^+ -H_2O$), 144 ($M^+ -CO$), 141 ($M^+ -CH_3O$) and 128 ($M^+ -CH_2CHOH$). Peak No. 8 gave major ions of m/z: 186 (M^+), 168 ($M^+ -H_2O$), 158 ($M^+ -CO$), 155 ($M^+ -CH_3O$) and 143 ($M^+ -CH_2CHO$). These compounds were identified as 8-oxo-methyl-octanoate and 9-oxo-methyl-nonanoate considering the results of Terao et al. [42]. The chief ions of peak No. 9 were m/z: 217 ($M^+ +1$), 185 ($M^+ -CH_3O$), 152 ($M^+ - 2xCH_3OH$) [89] and 143 ($M^+ -CH_2COOCH_3$). This fragmentation was the same as that of methyl esterified commercial nonanedioic acid. Peak No. 10 gave m/z: 224 (M^+), 206 ($M^+ - H_2O$), 193 ($M^+ -CH_3O$), 157 ($M^+ -CO=CHCH=CH$), 152 ($M^+ - CHCOOCH_3$) and 123 ($CH_3COOCH_2CH_2CH_2$) and was supposed to be 12-oxo-methyl-dodeca-9,11-dienoate.

These compounds were quantitatively determined on GC (Fig. III-3) and summarized as their free acids in Table III-4. Semialdehydes, hexanal, dicarboxylic acids and short chain monocarboxylic acids were major components of SP and occupied a half% of the lower molecular weight components.

TBARS in autoxidation products

The elution pattern from Sephadex LH-20 (Fig. II-2) observed with radioactivity was apparently different from the pattern detected by TBA test. Eventually, a broad elution

pattern was observed radiochemically and two obvious peaks were detected by the TBA test. TBARS in the former peak appeared to be mainly endoperoxides [78, 90] and alkylperoxides [91]. The major components of the latter peak are given in Table III-4. MA is generally considered a main product of lipid peroxidation and a major TBARS. An attempt was then made to quantitatively detect MA in SP. Most of the autoxidation products were insoluble in water and

Table III-4

Percent of Radioactivity in Components of SP

	Components	%
Polymers		36
Epoxyperoxides and endoperoxides		26
Lower molecular-weight components		38
1*	Hexanal	3.7
2	Hexanoic acid	0.15
3	Heptanoic acid	0.15
4	Octanoic acid	1.7
5	2,4-decadienoic acid	0.15**
6	8-oxo-octanoic acid	0.75**
7	3,5-decadienoic acid	0.29**
8	9-oxo-nonanoic acid	4.8**
9	nonanedioic acid	2.5
10	12-oxo-9,11-dodecadienoic acid	0.34**

*Numbers were on GC (Fig. III-3).

**These values were estimated using a quantity of commercial nonanedioic acid.

MA was very soluble [85]. A water extract of SP was obtained and the recovery was 1.5% radiochemically and 2.2% with the TBA test. This water extract was subjected to Sephadex G-10 gel filtration (Fig. III-5). Most of the water soluble materials was eluted in the early stage of chromatography as two big radioactive peaks, which were negative to the TBA reagent. On a shoulder of the latter peak, TBARS appeared as a single peak which agreed with the elution of authentic MA, while the TBARS occupied only 0.4%

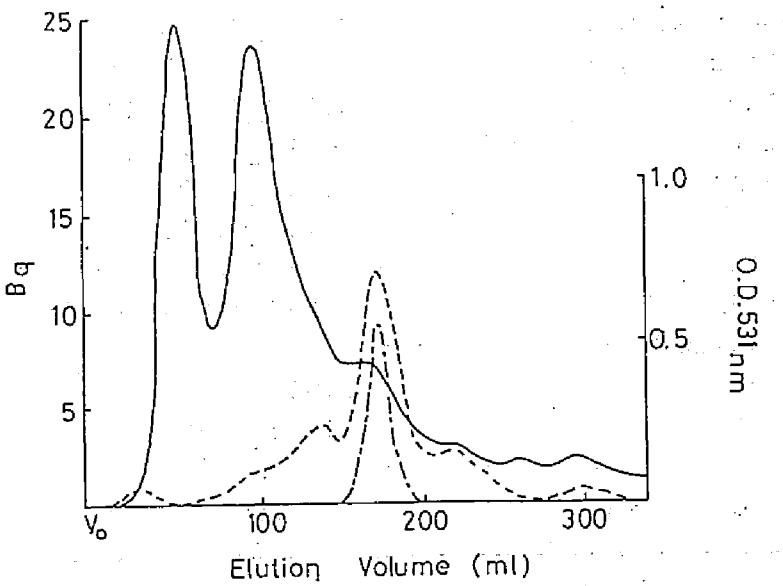


Fig. III-5. Detection of TBARS in the Water-Soluble Part of SP on Sephadex G-10 Gel Filtration. Two milliliters each of fractions (5 ml each) was assayed for radioactivity (———) and subjected to TBA test (----). The prepared authentic MA [92] was eluted and detected by TBA test (—·—·—). MA was observed at the elution volume between (V_o + 155) ml - (V_o + 190) ml.

or less in radioactivity of SP.

Color intensities of SP by the TBA test were compared with those of some TBARS (Table III-5). The color intensi-

Table III-5

Relative Color Intensities of SP and TBARS by TBA test

SP (51.3 kBq and 370 mg) was treated by several methods and colored by TBA test. The color intensities are expressed as the optical densities at 532 nm per mg of samples.

Sample	Optical density per mg (n)[a]
SP	0.181±0.015 (30)
Water-soluble part[b]	0.263±0.021 (3)
Filtrate from gel[c]	0.322±0.027 (3)
Water layer[d]	0.179±0.014 (4)
Distillate[e]	4.14±0.37 (4)
Reduced SP	0.016±0.002 (4)
LAHPO	0.788±0.035 (10)
Authentic MA	260±21 (30)
2,4-Decadienal	0.737±0.046 (10)
2,4-Nonadienal	0.826±0.174 (10)
2,4-Hexadienal	0.909±0.058 (10)
2-Hexenal	0.248±0.017 (10)
9-Oxononanoic acid	Y.C.[f]
Nonanedioic acid	ND

[a] Mean±SE with the determination number (n).
[b] Referred to [85].
[c] A part of eluate from Sephadex G-10 which agreed with the elution position of MA (Fig. III-5).
[d] The remaining part of aqueous suspension of SP after chloroform-methanol extraction.
[e] Followed the method of [93].
[f] Yellow color.

ties of the water-soluble part, filtrate and water layer of SP were the same as that of SP per se. Distillate of SP gave an intensity 20-fold the color of SP. MA are easily collected by distillation [93] or condensed by removing the nonpolar components with chloroform-methanol extraction. The MA-rich fraction can also be obtained by Sephadex G-10 gel filtration. However, color intensities of these treated SP were far from the color intensity of MA and close to that of LAHPO. These results demonstrate that the single peak detected by TBA test in Fig. III-5 could not be attributed to MA.

Marcuse and Johansson [94] reported that 2,4-alkadienals are typical TBARS. Color intensities of 2,4-decadienal, 2,4-nonadienal and 2,4-hexadienal by the TBA test were the same as that of LAHPO and close to those of the treated SP. Terao et al. [42] and Schieberle and Grosch [95] indicated that 2,4-decadienal are stoichiometically produced by the decomposition of 9-LAHPO (hydroperoxide at C_9 position). Matthews et al. [88] showed that 2,4-decadienal is unstable and is easily further oxidized. In the present analysis, 2,4-decadienoic acid , one of the further oxidized products was detected as given in Table III-4. The TBA reagent is especially colored by the aldehyde species [96]. The alkadienals in SP were further oxidized and the color

intensity of SP was less than that of LAHPO. Therefore, the peak of TBARS in Fig. III-5 may be attributed to these alkadienals, which are major TBARS in SP.

The color intensity of SP decreased considerably to 8.6% by reduction. Unstable compounds such as epoxyperoxides, endoperoxides and alkylperoxides as mentioned above were easily decomposed by reduction. TBARS were derived from these unstable compounds in SP [78, 90, 97] during the TBA test.

III-4 DISCUSSION

The composition of autoxidation products is changed by various conditions such as light energy, temperature, oxygen pressure, period, metal contaminants and so forth [41]. LAHPO content in the autoxidation products increased upon contact with the atmosphere (Table III-1 and 2). Next, an analysis was made of the composition of the products of [U-^{14}C]LA autoxidized under a certain condition. The autoxidation products consisted of 45% intact LA, 18% LAHPO, 13% a mixture of polymers, 9% epoxyperoxides or endoperoxides, 3% PP, 2.1% short chain carboxylic acid, 1.7% 9-ONA, 1.3% hexanal, 0.9% nonanedioic acid, 0.3% 8-oxooctanoic acid, 0.1% 12-oxododecadienoic acid and others (Table III-2 and 4, Fig.

III-1-4). Thus, unstable 2,4-alkadienals were the main intermediate products, but MA was not a major product of the autoxidation of LA.

In the field of medicine, the extent of lipid peroxidation has often been determined by the TBA test and the peroxide contents are expressed as the MA level [98-100]. An attempt was made to identify TBARS contained in the autoxidation products of LA. 2,4-Alkadienals were major TBARS but MA was not (Fig. III-5 and Table III-5). Frankel and Neff also reported that no correlation was found between the TBA value and analysis data of MA in the lipid peroxidation products [101]. TBA reagent reacts with aldehyde species. SP was a mixture mainly of aldehydes (Table III-3). 2,4-Alkadienals seem to be produced from LAHPO during TBA reaction [102]. Therefore, it was concluded that the TBA test was suitable for detection of SP but not LAHPO per se.

There have been many attempts to detect lipid peroxides, that is, observation of pentane and ethane exhaled by animals [103], measurement of singlet oxygen with chemiluminescence [104] and determination of peroxides per se by the iodine method [105], horseradish peroxidase [106], GC-MS [107] and fluorescent color [108]. Recently, a sensitive and simple method which uses the peroxidase activity of hemoglobin was

reported by the author et al. [109]. By coupling the reduction of hydroperoxides, endoperoxides and peroxy radicals to alcohols, the N-methylcarbamoyl derivative of methylene blue (leuco form) is oxidized and colored blue. This method has a high substrate specificity [110, 111] and its improvement is strongly recommended for application to biological samples.

IV TYPICAL EFFECTS OF AUTOXIDATION PRODUCTS ON PROTEINS

IV-1 INTRODUCTION

The autoxidation products damage proteins [112,113] and inactivate enzymes [114, 115]. Toxicity is due to polymerization of protein caused by free radicals of peroxides [116-118], modification of amino acid residues caused by aldehydes [119, 120], and oxidation of SH groups [121, 122]. Moreover, the autoxidation products react with the amino acid residues and form lipofuscin, which is considered to be a main factor of senescence [123-127]. Therefore, the damage to amino acid residues caused by the autoxidation products is an important problem.

Tappel and colleagues reported that all amino acid residues were destroyed by the autoxidation products [128, 129]. Gamage and Matsushita pointed out that methionine was mainly oxidized [130]. Braddock and Dugan [131], and Yanagita and Sugano [132] described the decomposition of lysine, histidine and methionine residues. However, the decomposition of amino acid residues by the autoxidation products has not been clarified.

In this Chapter, first is described a method for the removal of the autoxidation products from the reaction system

before amino acid analysis, and losses of amino acids were observed using lysozyme [133]. Next, incorporation of LAHPO and SP into casein molecule were measured. Furthermore, LAHPO and SP were administered orally to rats and their effects on gastric and intestinal mucous membranes were pathologically observed [134].

IV-2 MATERIALS AND METHODS

Materials. Lysozyme (6 x crystallized) and *Micrococcus lysodeikticus* were purchased from Seikagaku Kogyo Co. Hammarsten casein was used. LAHPO, SP and MA were prepared as described in Chapter III. PP was obtained by aging SP at 37°C for 20 months and the concentration was determined by titration with KOH.

Incubation of lysozyme with the autoxidation products. Incubation systems consisted of 5 mg of lysozyme in 1 ml phosphate buffer (0.1 M, pH 7.0) and 5 μmoles of reactants. These mixtures in spitch glasses (16.5x105 mm) were incubated. The system containing benzoyl peroxide (BP) was preincubated at 70°C for 1 h.

Methods removing autoxidation products from incubation

systems. The incubation mixtures were washed several times with 2 ml of diethyl ether. Lysozyme was precipitated by adding 4 ml of alcohol and centrifuging at 3800 rpm for 15 min. These precipitates were macerated and ground in 2 ml of a solvent (diethyl ether-alcohol=1:1) and further washed with this solvent several times. The former method of washing is referred to as Wash-Method I and all of these procedures are referred to as Wash-Method II.

Radioassay. Lysozyme was incubated with radioactive LA and 0.1 ml of the solution was placed on a filter paper disc (Whatman 3 MM). The paper was dried up and put in a vial. An aqueous scintillator (Chapter II) was used. The radioactive solution of casein was placed on a cellulose sheet and burned by a sample oxidizer.

Measurement of lysozyme activity. The lysozyme was washed by Method II and dissolved in 5 ml of potassium phosphate buffer (0.1 M, pH 7.1). An aliquot of 0.1 ml of lysozyme solution was added to a 3 ml buffer suspension of 0.5 mg *M. lysodeikticus.* Decrease in turbidity (measurement at 450 nm) was compared with that of non-incubated lysozyme [135].

Quantitative amino acid analysis. Both the incubation and washing of lysozyme were quantitatively performed and lysozyme was hydrolyzed in 6 N HCl at 110°C for 23 h. A part of the hydrolysate (1 mg of lysozyme) was submitted to an amino acid analyzer, Hitachi Model KLA-3A. Tryptophan residue was determined by hydrolysis in 6 N HCl containing 3% of purified thioglycollic acid [136]. These procedures, from the incubation to hydrolysis, were carried out in identical tubes. Recovery of amino acids was calculated by the HW method. Differences in these HW values from values of the corresponding amino acids of non-incubated lysozyme were expressed as % of loss.

Treatment with $NaBH_4$. The lysozyme precipitate was resuspended in 5 ml of water and the pH was adjusted to 9.5 with NaOH. Ten milligrams of $NaBH_4$ was added to the suspension and incubated at 37°C overnight. The reaction was stopped by the addition of acetic acid to pH 5 [82].

Digestion of casein by proteolytic enzymes. Casein was incubated with LA, LAHPO or SP and washed following the same procedures as in the case of lysozyme. The casein was digested by pepsin (at pH 2.0), trypsin (pH 7.6) and chymotrypsin (pH 8.2). Production of soluble peptides was

measured by the ninhydrin method.

Animals. Male Wistar albino rats, 5 weeks old and each weighing about 110 g (Clea Japan, Inc.), were housed for 1 week. The animals weighing 157-162 g were selected and separated into experimental groups of 10 rats each. Food was withheld for about 4 h and each rat was given one dose per day of LA, LAHPO and SP intragastrically at 6:30 pm using tuberculin syringes connected to the gastric tubes.

IV-3 RESULTS

Effects of the autoxidation products on activity of lysozyme

Fig. IV-1 shows changes in activity of lysozyme caused by the autoxidation products of LA. LAHPO and SP clearly inactivated lysozyme for the first 1 h. LA decreased the activity to 50% after 40 h. PP mildly affected lysozyme. The effects of the autoxidation products were in decreasing order as follows: SP > LAHPO > LA > PP.

Removal of autoxidation products from incubation systems

Lysozyme was incubated with [U-^{14}C]LA and washed by Wash-Method I or II. Fig. IV-2 shows the radioactivity remaining in the systems after washing. The radioactivity was easily

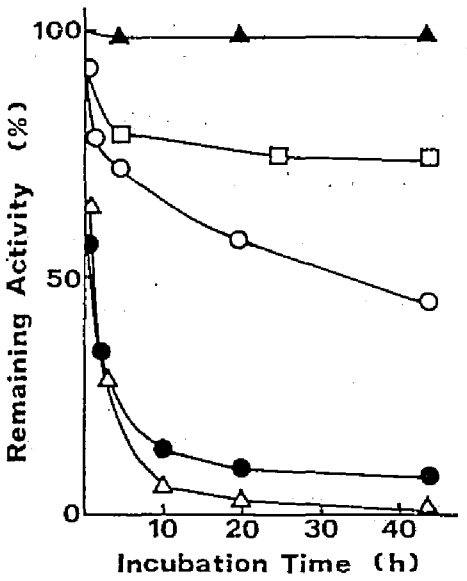


Fig. IV-1. Effects of the Autoxidation Products on the Lysozyme Activity. Lysozyme was incubated at 37°C with 5 μmoles of LA (—○—), LAHPO (—●—), SP (—△—), PP (—□—), and nothing (—▲—). The reaction mixtures were washed by Method II and the activity was measured.

removed from the lysozyme solution by Wash-Method I in the case of 1 h-incubation with [U-^{14}C]LA. However, when lysozyme was incubated for 8 days and washed by Method I, 20% of the radioactivity remained in the system. The lysozyme was precipitated with alcohol and the remaining radioactivity was decreased to 9%. The precipitate was further washed with diethyl ether-alcohol=1:1 (Wash-Method II) and the remaining activity became 3%. The lysozyme solution was

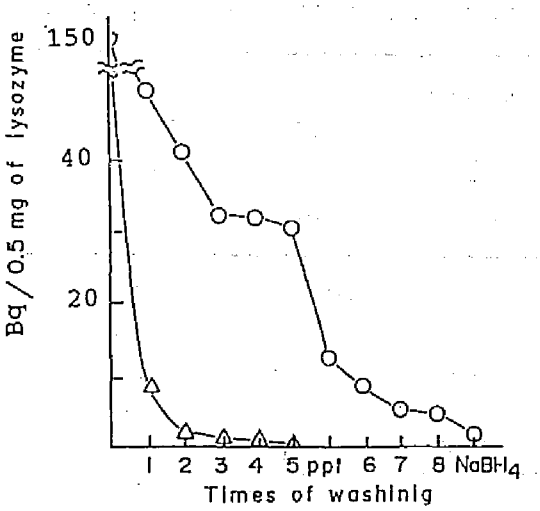


Fig. IV-2. Removal of Radioactivity from Incubation System of [U-^{14}C]LA and Lysozyme. Lysozyme was incubated at 37°C for 1 h (△) or for 8 days (○). The wash procedures from 1 to 5 followed Wash-Method I and from 1 to 8 Wash-Method II. The point at $NaBH_4$ indicates the treatment with $NaBH_4$ after Wash-Method II and the point at ppt indicates the precipitation of lysozyme with alcohol.

further treated with $NaBH_4$ and the remaining trace of radioactivity became 1%. Therefore, the autoxidation products could almost completely be removed from the reaction systems by Wash-Method II and treatment with $NaBH_4$.

Comparison of amino acid loss between Wash-Methods I and II

Lysozyme was incubated with LAHPO under a mild condition such as 37°C for 5 h and washed by Method I (Table IV-1). The loss of methionine residue was 70% and losses of 7 other

Table IV-1

Losses of Amino Acids of Lysozyme during Hydrolysis

Incubation with	LAHPO	LAHPO	LA	LAHPO
temperature (°C)	37	70	50	50
time (h)	5	8	31	31
Wash-Method	I	II	II	II
	loss %*			
Lys	12	12	0	4
His	24	5	3	3
Arg	10	0	0	0
Val	10	0	0	5
Met	70	0	0	7
Ileu	11	0	0	3
Leu	9	0	3	4
Tyr	10	0	0	0

*The other amino acids were not lost.

amino acid residues were 25-10%. Even when lysozyme was incubated under a drastic condition such as at 70°C for 8 h, no loss of amino acid residue was observed following Wash-Method II. It was concluded that LAHPO was not removed from the incubation system by Wash-Method I and might destroy the amino acids during hydrolysis. When lysozyme was incubated with LA and LAHPO at 50°C for 31 h and washed by Method II, LA did not cause any damage to the amino acid residues and LAHPO produced small damage.

Damage to amino acid residues caused by the autoxidation products

Table IV-2 shows the amino acid losses after long incubation with LA and LAHPO (at 45 °C for 100 days). All amino acids were lost at random. These losses did not seem to be caused by LA and LAHPO per se. LA and LAHPO were decomposed during the long incubation and their decomposed products might have attacked the amino acids. Lysozyme was incubated at 37°C for 8 days with the autoxidation products and three kinds of authentic reagents. The reaction mixtures were washed by Method II. Table IV-3 shows that mainly tryptophan, histidine and lysine residues were destroyed by the autoxidation products. LA damage was about 30% of tryptophan and histidine. LAHPO damage was 50% of tryptophan and histidine, and 20% of

Table IV-2

Amino Acid Losses during Incubation at 45°C for 100 Days

Incubation with	LA	LAHPO
	loss %	
Lys	32	51
His	43	50
Arg	12	11
Asp	5	18
Thr	9	20
Ser	0	16
Glu	14	17
Pro	24	13
Gly	13	14
Ala	12	14
1/2Cys	14	15
Val	14	14
Met	18	19
Ileu	14	27
Leu	14	13
Tyr	23	17
Phe	12	13

Table IV-3

Comparison of Amino Acid-Losses

by the Autoxidation Products and by Standard Reagents

Incubation with	LA	LAHPO	SP	PP	BP	MA	PA	SP-$NaBH_4$*
				loss %				
Try	30	56	95	38	88	67	0	91
Lys	11	17	73	7	21	36	5	76
His	37	42	67	17	58	22	14	65
Arg	7	9	14	11	23	11	0	18
Asp	0	0	0	0	17	0	0	0
Thr	5	5	9	0	17	18	11	9
Ser	0	0	4	0	9	18	5	4
Glu	4	4	17	0	17	6	14	8
Pro	0	-	0	0	35	8	9	11
Gly	4	0	0	0	20	0	0	0
Ala	5	0	0	0	21	0	0	0
1/2Cys	4	0	-	0	0	0	17	0
Val	7	6	17	0	19	0	0	7
Met	7	14	84	0	29	0	0	9
Ileu	5	0	8	0	19	0	0	7
Leu	0	0	5	3	21	0	0	6
Tyr	5	0	19	5	38	0	0	10
Phe	0	0	6	0	24	0	0	0

*Lysozyme incubated with SP was treated with $NaBH_4$ after Wash-Method II.

lysine. SP destroyed the tryptophan residue and 70% of lysine and histidine, 20% of tyrosine and so on. PP damage was 40% of tryptophan. Thus, SP was most reactive to lysozyme. The damages were in the order SP > LAHPO > PP or LA.

These destructive effects of the autoxidation products were compared with those of standard reagents. BP, MA and propionic acid (PA) were used as a standard radical reagent, dialdehyde and short chain carboxylic acid, respectively. The effect of BP on the amino acids was drastic and at random. Heterocyclic and aromatic compounds were suitable for radical trappers [137]. Particularly, tryptophan, histidine, proline and tyrosine were lost. The effect of BP was different from those of the autoxidation products. MA damaged mainly tryptophan, lysine and histidine similar to the autoxidation products. PA showed no destructive effect.

Oxidation of methionine residue of lysozyme by SP

The loss of methionine residue of lysozyme caused by SP was especially great (84%) (Table IV-3). The loss decreased to 9% when $NaBH_4$ was used (far right column). Free methionine was then incubated with SP and treated in the same way as lysozyme. Table IV-4 shows that methionine was oxidized to methionine sulfoxide, which was oxidized to

Table IV-4

Oxidation of Methionine by SP

Sample[a]		Treatment	Product (μmoles)[b]		
	μmoles		Met	$MetO_1$	$MetO_2$
Met	0.521	Non	0.520	ND	ND
	0.521	with SP for 8 days	ND	0.342	ND
	0.521	heated with SP[c]	0.519	ND	trace
$MetO_1$	0.654	Non	0.046	0.608	ND
	0.654	with SP for 8 days	ND	ND	0.543
	0.654	heated with SP	0.448	0.062	0.023
	0.654	treated with $NaBH_4$	0.050	0.612	ND
$MetO_2$	0.680	Non	ND	0.036	0.645
	0.680	with SP for 8 days	ND	ND	0.647
	0.680	heated with SP	0.013	0.710	ND
	0.680	treated with $NaBH_4$	ND	0.032	0.663
Lysozyme					
Met	0.702[d]	hydrolyzed with SP	0.484	ND	ND
	0.702	with reduced SP[e]	0.498	ND	ND

[a] Met, $MetO_1$ and $MetO_2$ are abbreviations for methionine, methionine sulfoxide and methionine sulfone, respectively.

[b] Products were determined by an amino acid analyzer.

[c] Samples were heated with 0.5 mM of SP (concentration as acetaldehyde) at 110°C for 23 h.

[d] Lysozyme, whose amount was 0.702 μmoles as methionine residue, was hydrolyzed with 0.5 mM of SP and the other amino acids were also almost intact.

[e] SP was reduced as described in Chapter III.

methionine sulfone during the 8-days of incubation with SP. Methionine sulfone was not oxidized by the incubation. During heating, which was the same as for the hydrolysis of lysozyme (at 110°C for 23 h), however, methionine sulfoxide was reduced by SP to methionine, and methionine sulfone to methionine sulfoxide. The treatment with $NaBH_4$ showed no change in both methionine sulfoxide and sulfone. When lysozyme was hydrolyzed in the system containing SP or the reduced SP, 30% of the methionine residues was lost. As described in Fig. IV-2, the trace amount of [U-^{14}C]LA remained in the reaction system after Wash-Method II and decreased markedly when treated with $NaBH_4$. SP might form a reversible complex with methionine residue during the incubation as reported by Tappel [138]. The complex was broken down by the hydrolysis and the methionine residue was lost, while the complex was easily decomposed by hydrogen gas generated from $NaBH_4$ and the methionine residue was protected against the oxidation during the hydrolysis.

Changes in digestibility of casein

Casein was incubated with SP under the same condition as lysozyme for 2 days. After Wash-Method II and treatment with $NaBH_4$ were carried out, no loss of amino acid residue was observed (Table IV-5). However, the digestibility of

Table IV-5

Losses of Amino Acid of Casein by Incubation with SP for 2 Days

	Wash-Method II	$NaBH_4$
	loss %*	
Lys	10	12
His	8	10
Asp	9	6
Thr	5	0
Ser	4	0
Glu	4	0
Ala	8	7
Met	39	5
Tyr	7	0

*The other amino acids were not lost.

casein by trypsin, chymotrypsin and pepsin was markedly decreased by incubation with the autoxidation products (Fig. IV-3 a-c). Although LA produced no change in the digestibility, LAHPO and SP easily deteriorated the digestibility. When casein was incubated with SP for 1 day, the casein could not be hydrolyzed by the proteolytic enzymes. Changes in solubility of casein by the autoxidation products were then measured (Fig. IV-4). SP insolubilized casein most easily. Next, incorporation of the autoxidation products into the casein molecule was

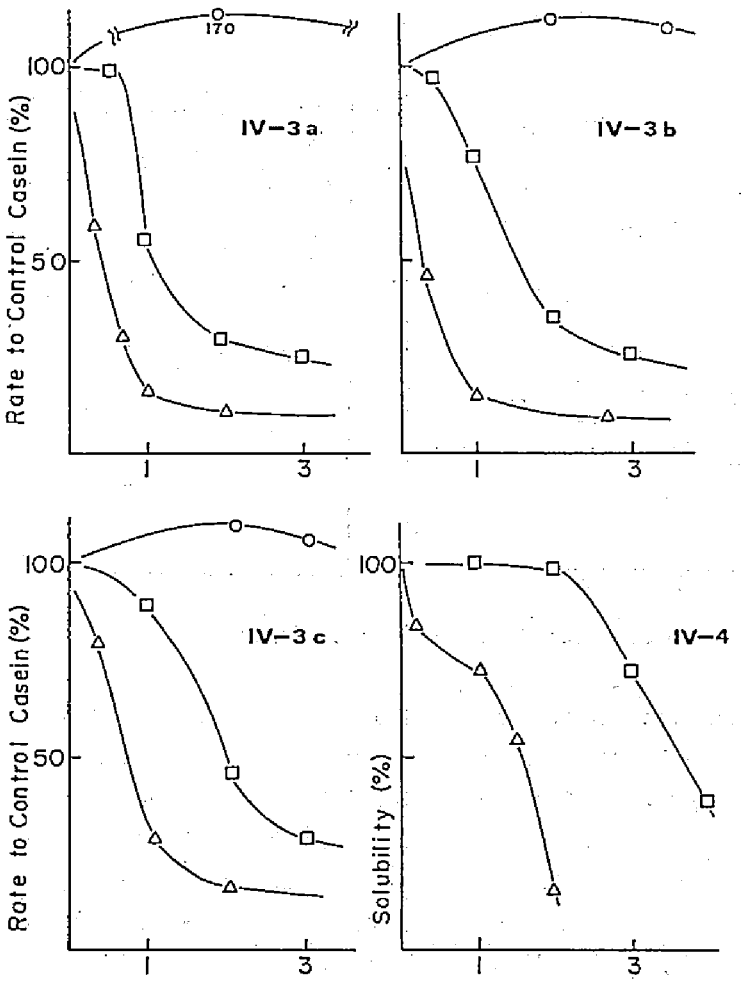
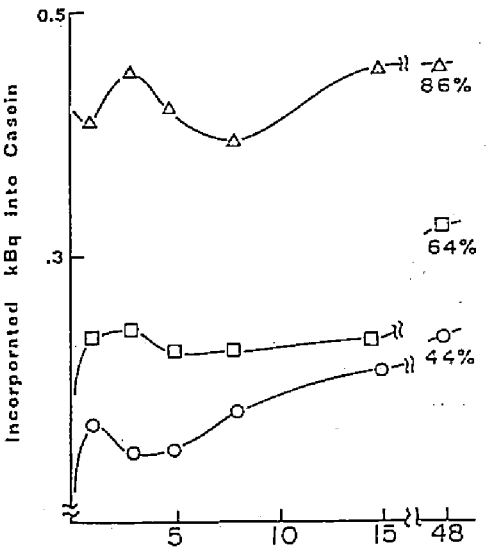


Fig. IV-3. Changes in Digestibility of Casein during Incubation with the Autoxidation Products. Incubation mixture consisted of 0.5% of casein, 0.1 M of Sörensen buffer (pH 6.5) and 5 mM of LA (○), LAHPO (□) or SP (△). After Wash-Method II was used, casein was mixed with 0.01% of trypsin (Fig. IV-3a), chymotrypsin (IV-3b) and pepsin (IV-3c).

Fig. IV-4. Changes in Solubility of Casein during the Incubation with LAHPO (□) and SP (△). After washing, precipitates of casein were dissolved in 0.05 N NaOH solution. The solutions were centrifuged at 3000 rpm for 10 min and soluble proteins in the supernatant were measured.



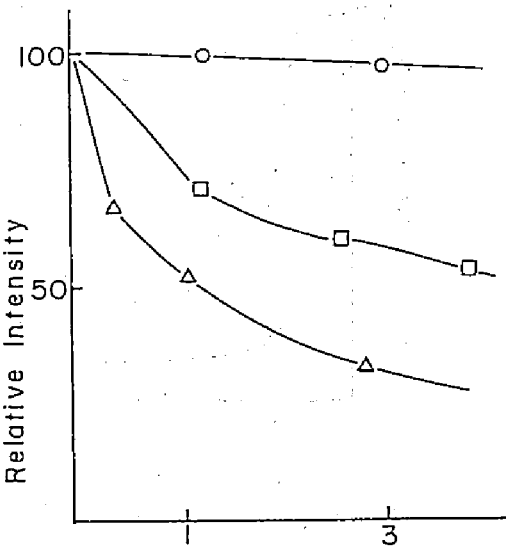


Fig. IV-5. Incorporation of Radioactive Autoxidation Products into Casein. Casein was incubated with 500 Bq and 5 mM of LA (○), LAHPO (□) and SP (△). After Wash-Method I, the radioactivity remaining in casein was measured. The % in figure shows the total incorporated amounts for 48 h incubation.

Fig. IV-6. Casein was incubated with nothing (○), LA (□) and SP (△). After Wash-Method I, casein solution was mixed with 2-p-toluidinyl-naphthalene-6-sulfonic acid and excited at 347 nm. Fluorescence was analyzed at 445 nm.

radiochemically observed. Casein was incubated with LA, LAHPO or SP and washed by Method I. Fig. IV-5 shows the radioactivity remaining in the casein molecule. LA, LAHPO and SP were easily incorporated into casein during 1 h-

incubation. After 48 h the incorporated amounts of LA, LAHPO and SP were 44, 64 and 86%, respectively. Furthermore, decreases in the hydrophobic pockets in casein molecule were observed with fluorescence of toluidinylnaphthalene sulfonic acid (Fig. IV-6). Both LA and SP seemed to be easily incorporated into casein pockets for a short time. These results indicated that SP influenced irreversibly a conformation of casein whereas LA did not. SP might particularly attack the basic amino acid residues.

Effects of autoxidation products on rat growth

LA, LAHPO and SP were administered orally to rats at several dosage levels (Fig. IV-7). When 700 mg/day of SP was given for 3 days, a remarkable depression in body weight occurred and resulted in death on the third day. Rats receiving a single dose of 700 mg of SP exhibited significant growth impairment ($P > 0.001$), reduction in food consumption and diarrhea on the first day. A single dose of 200-500 mg of LAHPO also gave diarrhea to rats, but did not suppress growth and produce anorexia. These animals receiving single doses recovered from the stress the following day.

No difference in body weight gain was detected among groups receiving no dose, the single dose of 250 mg of LA, 100 mg of LAHPO and SP, when a 0.05 probability level was

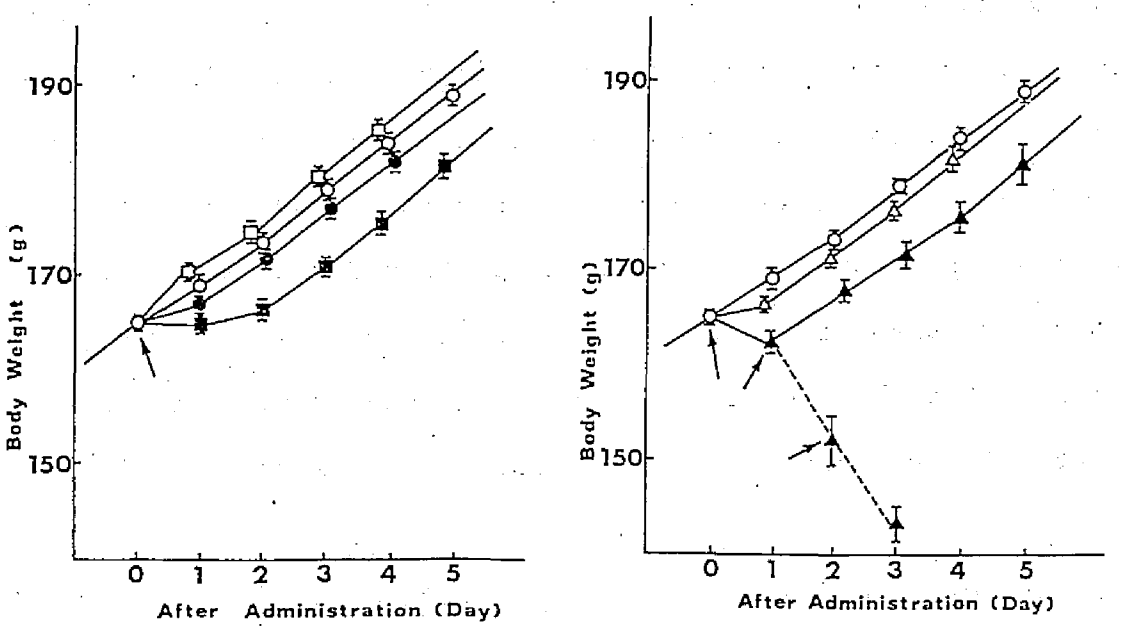


Fig. IV-7. Effects of LA, LAHPO and SP on Rat Growth. In the left figure, 110~140 mg of LA (○), 250~350 mg of LA (●), 75~120 mg of LAHPO (□) and 190~230 mg of LAHPO (■) were administered (indicated by arrow) orally to rats. In the right figure, none (○), 100~150 mg of SP (△) and 650~750 mg per day of SP (▲) were administered (indicated by arrows).

chosen. All of these animals were clinically normal.

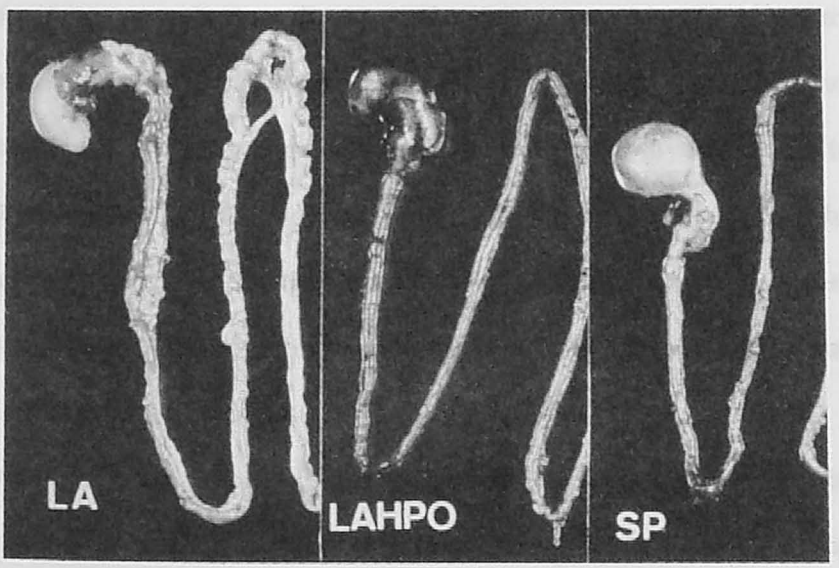
Pathological Observation of Toxicity of SP

The rats receiving 3 consecutive doses of SP were submitted to a pathological anatomy on the third day. No abnormality was found in the heart, spleen, liver, kidney and testis. Hemorrhage was found mainly in the gastro-intestines (Table IV-6). LAHPO rats had also congestion in the

Table IV-6

Hemorrhage Caused by Autoxidation Products Given Orally

Rats given	LA	LAHPO		SP					
				killed			dead		
		I	II	I	II	III	I	II	III
				Hemorrhage					
Lung	-	+	+	-	-	+	+++	+++	+++
Stomach	-	+	+	+++	+++	+++	++	+	+
Duodenum	-	+	+	+	+	+	+	+	+
Jejunum	-	+	+	+	+	+	+	+	+
Cecum	-	+	+	+	+	+	+	+++	+++
Colon	-	+	+	+	+	+	+	+	+
Rectum	-	+	+	+	+	+	+	+	+



Photograph IV-1

Animal Gastro-intestines Given LA, LAHPO and SP

gastro intestinal tract and LA rats had no hemorrhage. The rats receiving SP showed inflation near cardia in stomach, whose diameter was about 1 cm (Photograph IV-1). Retention of undigested food was noticed in the stomach and edema was also observed on mucosa. Thus, SP was most toxic to the animal gastrointestinal tract.

IV-4 DISCUSSION

Interactions between the autoxidation products and proteins were investigated using lysozyme and casein. Lysozyme is a stable enzyme and much analytical data has been reported by Karel and colleagues [139-141]. Losses of amino acids in casein are also known [142].

When lysozyme was incubated with LA for 8 days, LA could not be removed from lysozyme by an ether wash, Wash-Method I (Fig. IV-1). The autoxidation products were also easily incorporated into casein in the following order: SP > LAHPO > LA (Fig. IV-5 and 6). These autoxidation products remaining in the protein seemed to decompose amino acids during the hydrolysis (Table IV-1). However, almost all of the incorporated products were removed by alcohol precipitation (Wash-Method II) and $NaBH_4$ treatment (Fig. IV-1). After washing by Method II and $NaBH_4$ treatment, the losses of amino acids in lysozyme were measured (Table IV-3). Mainly

tryptophan, lysine and histidine were lost and the extent of losses caused by the autoxidation products followed the order, SP > LAHPO > LA ≧ PP. LA and LAHPO were autoxidized to SP during incubations described in Chapter III and then seemed to react with the amino acid residues (Table IV-2 and 3). The destructive effects of SP and LAHPO were similar to that of MA but not BP (Table IV-3). Therefore, the toxicity of autoxidation products to protein seems to be caused by aldehydes, which are the major components of SP (Chapter III).

The above data clarifies the interaction between autoxidation products and protein as follows: The autoxidation products were easily incorporated into the protein and changed the conformation of protein. The protein was subsequently insolubilized (Fig. IV-3 and 4, Table IV-5) and then, Schiff bases were formed by the aldehyde species mainly with the basic amino acids [143-146]. Tryptophan, lysine and histidine were particularly destroyed by SP, while methionine and cystein were rather easily oxidized [147, 148].

Furthermore, the toxicity of autoxidation products to rats was observed. The autoxidation products were administered intragastrically and were detrimental to rat growth in the following order: SP > LAHPO > LA (Fig.IV-7). The rats

given 3 consecutive doses of SP died and the cause seems to have been impairment of the gastro-intestinal mucosa (Table IV-6 and Photograph IV-1). Therefore, when a large amount of SP was administered orally, aldehydes injured the mucous protein of the gastro-intestinal tract, inhibited intestinal absorption, and subsequently killed the animals.

Hundred milligrams of autoxidation products had no influence on the rat growth (Fig. IV-7). These autoxidation products might be incorporated into the body and the aldehydes might induce serious diseases [149] and senescence [8, 126]. This problem is discussed in the next Chapter.

V ABSORPTION OF AUTOXIDATION PRODUCTS ORALLY ADMINISTERED INTO RAT BODY

V-1 INTRODUCTION

The significant biological and deleterious roles of lipid peroxides in vivo have been well recognized [1-5]. Exogenous lipid peroxides may also contribute to diseases [150] and stimulate endogenous peroxidation [151]. Animals suffer various injuries by intakes of thermal polymerized oils [152, 153] or the autoxidized oils [154-158]. Measurement of the absorption of exogenous peroxidation products into body is important problem in a field of nutrition and may clarify a relationship between the oral intakes of autoxidation products and serious diseases [159]. The intakes of large amount of autoxidation products gave diarrhea to the animals (Chapter IV). In this Chapter, 100 mg each of LA, LAHPO and SP were administered intragastrically and the extent of incorporation was radiochemically made clear using materials uniformly labeled with ^{14}C [134].

Studies on the gastrointestinal absorption of LA [160] and LAHPO [161-165] have been done. Then, the extent of incorporation of SP was compared with those of LA and LAHPO. Further, the effect of SP on liver was examined.

V-2 MATERIALS AND METHODS

Autoxidation products of [U-^{14}C]LA. [U-^{14}C]LA of 172 kBq/mmol was used. The autoxidation products were obtained and characterized as described in Chapter III.

Animals. Male Wistar albino rats, 5 weeks old and each weighing about 110 g (Clea Japan, Inc.), were housed at approximately 23°C with a light and dark cycle of 12 h. The diet was prepared daily and its PV was maintained at less than 0.5 meq/kg. It consisted of 30% sucrose, 24% cornstarch, 25% casein, 15% soybean oil, 4% McCollum's salts mixture, 1% cellulose powder, 1% vitamin mixture. The vitamin mixture was purchased from Shionogi and Co., Ltd. and the solution of 1 ml contained 2500 IU retinyl palmitate, 2.5 mg thiamine hydrochloride, 3.5 mg riboflavin phosphate, 2.5 mg pyridine hydrochloride, 25 mg nicotinamide, 10 mg panthenol, 75 mg ascorbic acid, 250 IU calciferol and 100 mg lysine hydrochloride. After feeding for 1 week, the animals weighing 157-162 g were selected and separated into experimental groups. Food was withheld for about 4 h and each rat was given a single dose of LA, LAHPO or SP intragastrically at about 6:30 pm using tuberculin syringes connected to the

gastric tubes. The amounts administered were determined by measuring the radioactivity remaining in the syringes. The radioactivity administered to each rat was converted to a standard of 46.4 kBq. The rats were then placed individually in sealed glass boxes (metabolism cages) and given diet and water ad libitum. Air from the box was slowly removed by a vacuum pump into a bottle containing 25 ml of monoethanolamine.

Radioassay of animal excreta. The monoethanolamine bottle trapping ^{14}C was changed at regular intervals. In triplicate, 3 ml of the monoethanolamine was transferred into a vial and dissolved with 9 ml methanol in 8 ml of nonaqueous scintillator (Chapter II). Urine was washed periodically into a beaker and absorbed by cellulose sheets. Feces were also collected on cellulose sheets. These sheets were weighed and burned by a sample oxidizer (Packard Model 305 Tri-Carb). The counting efficiency of each vial was determined by an external standard. Recovery of radioactivity with the sample oxidizer was 97.2±1.2% (n=10). A higher efficiency was obtained by this procedure than by the direct use of a scintillator containing solubilizer.

Measurement of radioactivity incorporated into animal

tissues and organs. The radioactive SP (46.4 kBq each) was administered orally to 40 rats. Five rats were picked at random every regular interval after administration, stunned by a cephalic blow and bled from the carotid artery. Tissues and organs were excised, perfused or washed with saline solution, weighed, and cut into small pieces. A sample of these pieces, in triplicate, was wrapped with a cellulose sheet and burned by the sample oxidizer. Some of the hepatic pieces were examined by the TBA test, while blood was assayed for transaminase activities by the Karmen method [166].

Isolation of hepatic mitochondria and microsomes. The hepatic pieces were homogenized with 10 volumes of 0.25 M sucrose containing 1 mM ethylenediaminetetraacetate and 1 mM tris buffer (pH 7.4) in a Teflon homogenizer. After initial centrifugation for 10 min at 700 x g the supernatant fraction was subjected to further centrifugation for 10 min at 12,000 x g. The supernatant was submitted to isolation of microsomes and the pellet was resuspended to the original volume in the above sucrose. After further centrifugation for 15 min at 9000 x g the pellet was resuspended in 150 mM of KCl and was used for experiments as the mitochondria fraction. The supernatant at 12,000 x g was recentrifuged at 24,000 x

g. The supernatant fraction was subjected to further centrifugation at 54,000 x g and the pellet was resuspended in 150 mM of KCl and then, was referred to the microsomes fraction. The fractions of mitochondria and microsomes were added to each 2.0 mg of BHT. After the centrifugation at 105,000 x g of the supernatant at 54,000 x g, the supernatant was used as cytosol fraction. Nitrogen contents in these fractions were measured by the Kjeldahl method.

TBA test. A 2 ml aliquot of liver homogenate was submitted to the TBA test as described in Chapter III. The difference in the optical density from that of nontreated rats was presented as a μeq value of MA after conversion, with a calibration curve of authentic MA. The amounts of TBARS in feces (wet weight) and in urine (color intensity of creatinine by Jaffe reaction) were also measured.

Statistical analysis. The Student's t test was used to determine statistical significance. The variability of the data is presented as mean±SE.

V-3 RESULTS

Comparison of excretion of radioactive substances by SP-, LA-

and LAHPO-fed groups

Fig. V-1 shows fecal excretion of radioactive substances from SP-, LA- and LAHPO-fed groups. These three groups exhibited similar excretion patterns (peaks at 20 h after administration), but the SP group had a secondary minor peak

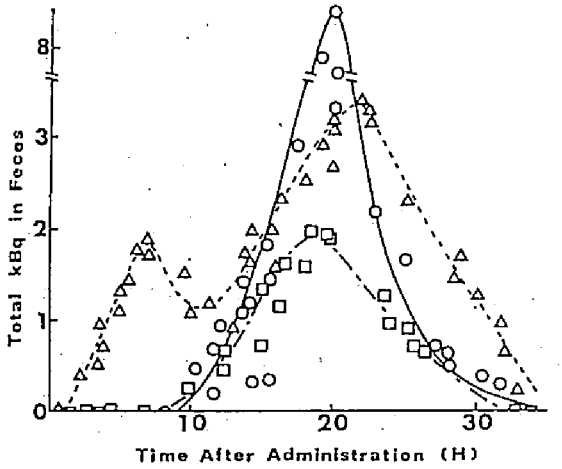


Fig. V-1. Changes in Radioactivity in Feces of Rats Orally Administered Labeled LA, LAHPO and SP. The rats were divided into 3 groups of 5 rats each and given doses of LA (——○——), LAHPO (-·-□-·-) and SP (----△----). The feces of each rat were collected at 4.3, 5.5, 8.5, 13, 17, 20, 23, 29 and 34.5 h after administration. Mean value±SE of the radioactivity of feces were calculated and plotted at each midpoint of the sampling times.

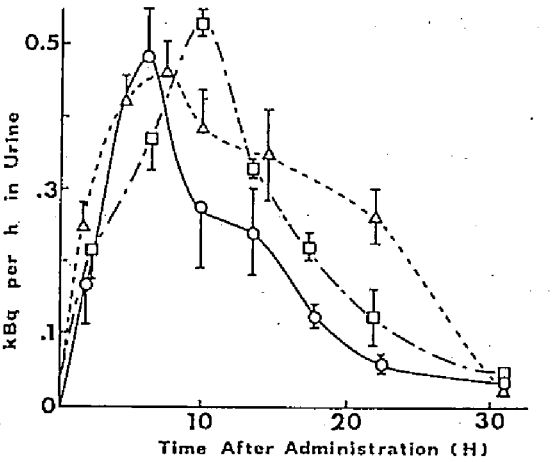


Fig. V-2. Changes in Radioactivity in Urine of Rats Orally Administered Labeled LA (——○——), LAHPO (-·-□-·-) and SP (----△----). The radioactvity in urine of each rat was assayed at about 4 h intervals until 24 h and at 38 h after the administration. The activity was divided by the interval times (h) and plotted at each midpoint.

at 7 h. The amount of TBARS per radioactivity in the minor peak was 0.154±0.015 μeq/7240 Bq, while the amount in SP per se was 0.151 μeq/7240 Bq. Although diarrhea was never observed in the SP group, this ratio indicated that the minor peak was excreted SP per se. Fig. V-2 shows that urinary excretion patterns of radioactive substances of these 3 groups were similar to each other. The PV in the urine of the LAHPO group was negative. A small amount of TBARS was detected in the urine of the SP group. Fig. V-3 shows radiorespirometric patterns of 3 groups. The SP group had

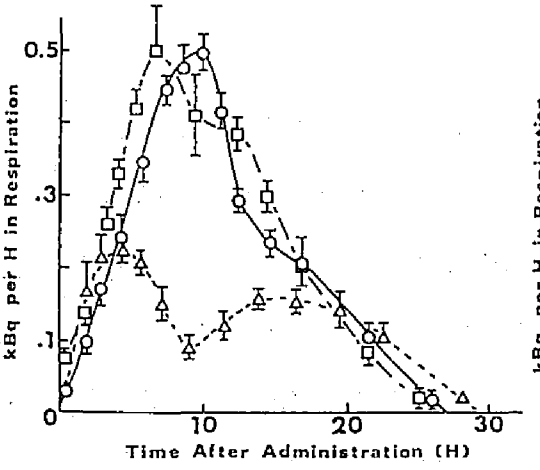


Fig. V-3. Radiorespirometric Patterns of Rats Orally Administered Labeled LA (—○—), LAHPO (-·-□-·-) and SP (---△---). The respiratory radioactivity was measured at regular intervals, divided by the interval times (h) and plotted.

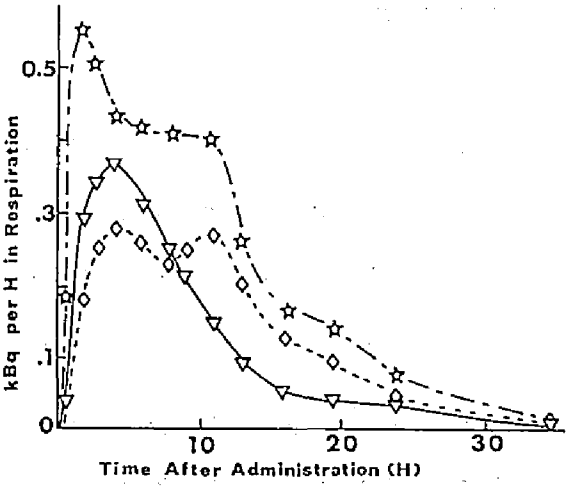


Fig. V-4. Radiorespirometric Patterns of SP-H (—▽—), SP-L (----◇----) and SP-R (-·-☆-·-). The time course was observed following the same procedure as in the case of SP.

an expiration rate with 2 peaks around 5 and 15 h, whereas the LA and LAHPO groups indicated single peaks at 10 and 7 h, respectively. Then SP was separated by Sephadex LH-20 into molecular weight components higher (SP-H) and lower (SP-L) than the molecular weight of LAHPO (Fig. III-2 and Table III-4), and it was also reduced by $NaBH_4$ (SP-R) (Table III-5). When SP-H, SP-L and SP-R were administered orally to 2 rats each, the radiorespirometric pattern of the SP-L rats tended to differ from that of the SP-H rats (Fig. V-4). The SP-H rats gave a single peak at 4 h, and the SP-L rats gave 2 peaks, at 4 h and 12 h. The SP-R rats excreted $^{14}CO_2$ faster than the above rats.

Total percentage of radioactivity incorporated into body

The total radioactivity excreted by each rat until 90 h after administration was calculated (Table V-1), because a low level of radioactivity could be detected in excreta of all rats even after 75 h. The amounts of radioactive substances excreted through feces by the SP group was larger than that from any other group. When administered radioactivity minus excreted activity to feces was defined as the amount of radioactive substances retained in the body, these incorporated substances were 95% in the LA, 85% in the LAHPO and 55% in the SP groups. Discharge of radioactive sub-

Table V-1

Total Radioactivity Recovered from Excreta

	Administered of:					
	LA	LAHPO	SP	SP-H	SP-L	SP-R
	Mean±SE with 5 rats			Mean of 2 rats		
mg of feces	2120±980	2250±300	5860±220	1730	2030	3810
Recovered % of administered amount from feces	6.4±1.7	13.4±1.9	45.3±1.0	23	18	25
Recovered % of the respective absorbed amount into body from urine	17.2±2.7	29.3±1.8	51.9±0.8	22	38	24
respiration	22.4±1.9	23.9±1.2	22.9±0.7	16	17	31

Radioactivity from feces, urine and respiration excreted by each rat (Fig. V-1) until 90 h after administration was totaled.

stances by urination increased significantly in the order of SP > LAHPO > LA groups with the SP group excreting about half of the activity incorporated into body. The production of $^{14}CO_2$ was proportionately the same (25%) for every group. No remarkable differences were observed among the excretion percentages of SP-H, SP-L and SP-R rats.

Incorporation of radioactive substances into liver in SP group

Table

Incorporation Amount of Radioactivity from Orally

	Mean±SE of Bq (n=5)		
	H after		
	0.5	3	6
Brain	51±9	44±2	79±14
Lung	49±6	60±5	88±7
Heart	17±1	15±1	30±3
Blood(ml)	10±2	166±18	31±3
Stomach	1080±130	455±84	341±145
Gastric content	35470±6493	23249±3406	13273±155
Intestine	295±47	508±85	903±153
Intestinal content	894±311	4242±551	7375±663
Pancreas	26±5	25±2	23±4
Liver	397±43	632±65	960±25
Spleen	16±2	12±1	47±18
Kidney	77±5	73±2	143±24
Perirenal fat pad	63±13	60±7	65±14
Epididymal fat pad	49±16	52±11	60±6
Testis	44±5	31±4	84±13
Femoral	32±2	30±6	32±3

In 96 h rats, only liver was radioassayed. The

V-2

Administered SP into Rat Organs and Tissues

per whole organ or tissue

administration			
12	24	48	72
55±9	72±7	56±6	35±4
53±1	114±13	86±10	48±11
20±1	20±1	20±2	19±1
57±6	184±25	32±1	47±3
120±47	150±18	66±12	14±1
12050±1629	881±198	126±23	50±15
437±80	624±65	192±19	67±7
4473±531	4412±508	2136±264	560±55
22±2	35±6	24±2	13±1
1212±61	1179±102	323±26	168±14
21±1	26±3	22±2	10±1
78±12	92±7	60±3	54±2
90±9	103±24	81±4	68±4
89±9	109±22	63±3	47±4
50±7	56±4	41±5	29±4
28±6	35±9	24±2	19±2

results are shown in Fig. V-5.

Rats given single doses of SP were exsanguinated at regular intervals and radioactivity in the tissues and organs was measured (Table V-2). The radioactive contents in intestinal lumen, which probably were SP, increased with a decrease in radioactive contents in gastric lumen. The activity of the intestinal contents reached a maximum at 6 h after administration and decreased to 1% of the given total at 72 h. The change in radioactivity of blood exhibited 2 peaks at 3 h and 24 h. Incorporation of the radioactivity into the intestine also exhibited 2 peaks, a major one at 6 h and a secondary one at 24 h. Changes in the incorporated radioactivity in brain, lung, spleen, kidney and testis were parallel to that in intestine. The radioactivity in fat pads, pancreas and femoral muscle gave a peak at 24 h. In liver, the radioactivity clearly increased and reached a maximum at around 12 h. The quantity in liver was 2.6% of the dose and the greatest of all tissues and organs.

Hepatic mitochondria and microsomes were isolated from the cytosolic fraction. Recovery % of nitrogen into each fraction had no variation (Table V-3). Then, the radioactivity in these cellular fractions were measured. The radioactivity increased with time after administration in both mitochondria and microsomes, while activity in the cytosolic fraction reached a maximum at 12 h. The accumula-

Table V-3

Incorporation of Radioactivity into Hepatic Mitochondria and Microsome of SP-fed Rat

H after administration	Mean±SE of Bq (n=4)			
	Whole liver	Mitochondria	Microsome	Cytosol
3h-SP	424±54	54±5	36±4	326±24
7h-SP	669±63	52±7	33±4	247±5
12h-SP	1982±228	188±16	142±24	540±15
24h-SP	1636±156	210±16	146±15	297±18
7h-LA	1769±229	156±12	126±1	814±89
7h-LAHPO	1851±262	157±4	124±17	889±74
Recovery of N	100%	18.2±0.3%	7.98±0.24%	24.1±0.3%

tion ratio of radioactivity were about 10% in mitochondria and microsomes and were almost constant. These ratios were the same as in the case of LA- and LAHPO-fed groups. Therefore, it was considered that the orally given SP was incorporated into hepatic mitochondria and microsomes.

Effects of orally administered SP on liver

A change in hepatic lipid peroxide content was observed with the TBA test (Fig. V-5). The lipid peroxide content elevated with the increase in radioactivity until 24 h. After rapid decline of the radioactivity, the lipid peroxide

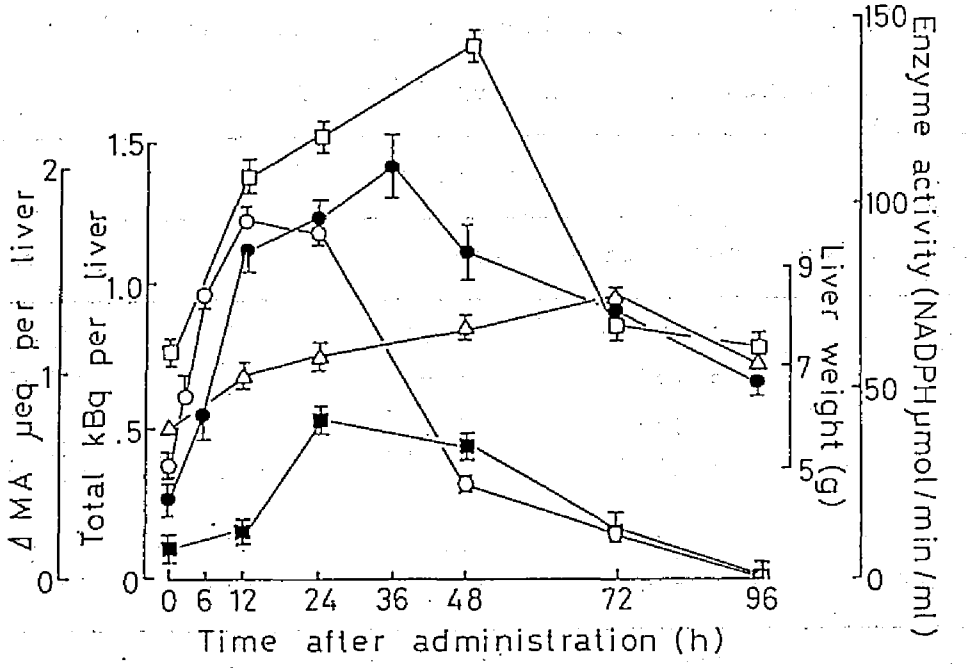


Fig. V-5. Effect of Oral Intake of SP on Liver. The radioactivity incorporated into liver (—○—) is given in Table V-2. Liver weight (—△—), lipid peroxide content (—●—), serum glutamic oxaloacetic transaminase (—□—) and glutamic pyruvic transaminase (—■—) were measured concurrently with assay of radioactivity incorporation.

content still remained at the high level, which was accompanied by increases in serum glutamic oxaloacetic transaminase and glutamic pyruvic transaminase activities and by a slight hypertrophy (about 1.5-fold; $P < 0.001$).

Various amounts of SP were administered and changes in the incorporated amounts into liver were observed after 12 h. Table V-4 shows that the ratio decreased noticeably from 2.5% to 1% with increase of the administered amount from 100 mg to

Table V-4

Incorporated Amounts of Radioactivity into Liver after 12 h When Various Amounts of SP were Given Orally

Given mg	Incorporated into liver mg*	(%)
115	3.00	(2.6)**
230	4.03	(1.8)
300	4.29	(1.4)
410	4.63	(1.1)
570	4.90	(0.9)

*These amounts were calculated from their radioactivity.
**See in Table V-2.

570 mg. Thus, the accumulation of radioactive substances of SP was 3-5 mg in liver.

V-4 DISCUSSION

The SP-administered group excreted about half of the given amount through feces (Table V-1). The total fecal weight was 3-times the weight of LA group. SP seemed to have an effect of exfoliation on brush border membrane such as that exhibited by some kinds of detergents [168]. The remaining half was incorporated into the body, and 1/2 was discharged by urination and 1/4 was exhaled. Thus, radio-

active substances in the SP group were metabolized to CO_2, as also seen in the LA and LAHPO groups.

While the rates of excretion through both feces and urine in the SP group were almost the same as those in the LA and LAHPO groups (Fig. V-1 and 2), the radiorespirometric pattern in the SP group was different from those in the other groups (Fig. V-3). The radiorespirometric pattern in the SP group gave 2 peaks and was similar to that in the SP-L rats (Fig. V-4). SP-L might comprise 2 components. One was rapidly metabolized to CO_2, while the other was not.

SP-H was composed mainly of polymers of LA (Table III-4). The molecular-weight range was determined to be about 300-1000 by Sephadex LH-20 gel permeation (Fig. III-2). No remarkable differences were observed in either respiration of excretion between SP-H and SP-L rats (Table V-1). It is believed that SP-H could be absorbed by the rats, although thermally oxidized oil which was the higher molecular-weight polymer was not [169].

It was made clear that SP was toxic to protein in Chapter IV. The present data demonstrate that 1/2 radioactivity of the orally administered SP is incorporated into the rat body, where 1/2 is excreted through urine and 1/4 through CO_2 (Table V-1). The remaining radioactivity was accumulated mainly in liver after 12-24 h (Table V-2) and capacity of the

accumulation was about 5 mg of SP (Table V-4). The radioactivity was incorporated in mitochondria and microsomes (Table V-3) in the rodent liver.

The accumulated materials affected deleteriously on the hepatic condition (Fig. V-5). The lipid peroxide content in liver increased by the intake of SP and remained at a high level even after the radioactivity of SP disappeared. Moreover, elevation of serum transaminase activities and a hypertrophy of liver were also detected.

On the other hand, the accumulation peak of radioactivity in tissues and organs was in agreement with the second peak of the radiorespirometric pattern. The incorporated SP might be partly unchanged, metabolized in liver, contributed to hepatic impairment and then was transferred to the other tissues and organs. The problem whether the autoxidation products are unchanged or not and the metabolism of incorporated substances in liver will describe in the next Chapter.

VI FATE OF SECONDARY AUTOXIDATION PRODUCTS IN LIVER

VI-1 INTRODUCTION

The Chapter V made clear that the radioactive substances of [U-^{14}C]LA, LAHPO and SP were incorporated into rat bodies to the extent of 95%, 85% and 55% respectively. Then, an interesting problem is whether the autoxidation products are incorporated unchanged in form or not.

The incorporation of LAHPO had been investigated by many workers [161-165]. Holman and Greenberg [170] demonstrated that the intraperitoneal LD_{50} of ethyl linoleate hydroperoxide for mice is 12 mg and that the oral dose does not kill. Bergan and Draper [171] reported that oral [1-^{14}C]methyl linoleate hydroperoxide is not absorbed per se into the animal body. Nakatsugawa and Kaneda [172] showed that only 0.23% of methyl linoleate hydroperoxide in the orally administered total is detected in rabbit lymph by high performance liquid chromatography. Thus, it is believed that orally fed LAHPO is readily decomposed to SP [161-163] or reduced to nontoxic substances such as hydroxy FA [164, 165, 171] in the animal gastrointestinal tract. On the other hand, polymers may be absorbed into lymph [173] and the low molecular-weight components in SP may be easily absorbed

into body.

Three daily consecutive doses of SP resulted in the death of animals on the third day (Chapter V). Although the single dose of SP produced the stress in the animals on the first day, the animals easily recovered. Furthermore, the dose of smaller amount of SP had no effect on the animals, even when administered orally or through intraperitoneal injections. SP may be absorbed unchanged in form and detoxified. Therefore, biological fate of the absorbed substances of SP must be made clear.

The radioactivity of SP was accumulated in liver. In this Chapter, attempts were made to identify SP per se in liver and to clarify the metabolism of absorbed substances [174].

VI-2 MATERIALS AND METHODS

Animals. Six-weeks old rats were used after feeding on the prepared diet for 1 week as described in Chapter V. LAHPO and SP were obtained from 172 kBq/mmol of the autoxidized [U-^{14}C]LA (Chapter III). Three rats were intragastrically received 100 mg each of LAHPO and exsanguinated after 2.5 h. Blood was collected into 100 ml of saline solution. Groups of 5 rats each were given 560 mg each of

SP. The blood and liver of these rats were isolated.

Separation of lipid in blood and liver. Hematic and hepatic lipids were analyzed. Blood was homogenized with 100 ml of a chloroform-methanol=2:1 solvent and 2 mg of BHT, twice. The hepatic mitochondria and microsomes were isolated (Chapter V) and homogenized with the chloroform-methanol=1:2 and BHT. The chloroform layer was obtained by centrifugation (3000 rpm, for 10 min). These extracts were evaporated, dissolved again into chloroform and applied on a 1x17 cm column of silica gel (Wakogel C-100). The chromatography followed the method of Rouser et al. [175]. Two hundred milliliters of chloroform, 800 ml of acetone and 200 ml of methanol were eluted in succession. The chloroform eluate, which was a fraction of neutral lipids, was further applied on TLC. TLC was developed with a solvent system of hexane-diethyl ether-acetic acid=80:20:1. Bands of triglycerides, free FA, diglycerides, monoglycerides and steroid esters were individually scraped off the plate. These lipids were extracted by chloroform and assayed radiochemically.

Gel permeation chromatography of blood. The chloroform extract of blood was evaporated and suspended in 5 ml of

water and applied on a 2.5x92 cm column of Sephadex G-10 whose void volume was 175 ml. The chromatography was carried out in accordance with the method for MA (Chapter III). Half of each fraction (8 ml) was submitted to radioassay and the other half to the TBA test.

GC-MS analysis. The samples were methylesterified and applied to GC-MS with a 1 m column of Silicon OV-1 (2%) on gas chrom Q (60-80 mesh) after BHT was added as an internal standard. The analytical conditions were elevation of the temperature from 60°C to 250°C at 10°C/min; flow pressure of helium gas of 1 kg; ionic voltage of 20 eV; and a sample temperature of 100°C and a chamber temperature of 200°C. These conditions were almost the same as those for the analysis of the low molecular weight components of SP (Chapter III).

Preparation of hydroperoxides of phosphatidylethanolamine and trilinolein. Phosphatidylethanolamine and trilinolein were purchased from Sigma Chem. Co., Ltd. and Nakarai Chem. Co., Ltd. Their hydroperoxides were prepared with photosensitized oxidation reported by Terao et al. [21].

Measurement of hepatic enzyme activity. Activities of 7

kinds of cytosolic enzymes were measured. Glutathione peroxidase activity was assayed with the method of NADPH disappearance [176] using LAHPO as the substrate. Catalase activity was determined by the method of Chance [177] using H_2O_2. Superoxide dismutase activity was measured by reduction of nitroblue tetrazolium [178]. Aldehyde dehydrogenase (NADP dependent type) [179], phosphofuructokinase [180], hexokinase [181] and glucose-6-phosphate dehydrogenase [182] activities were assayed by the changes in NADH or NADPH. Five kinds of mitochondrial enzymes were also measured. Isocitrate dehydrogenase activity [183] was assayed by the NADPH-method. Succinate dehydrogenase assay [184] used a potassium ferricyanide method. Cytochrome C oxidase activity was measured with oxidation of cytochrome C [185]. Aldehyde dehydrogenase activity of NAD-dependent type was also measured [179]. The activity of carnitine palmitoyl CoA transferase was determined by the amount of CoA-SH [186]. Microsomal enzyme, acetyl CoA carboxylase activity was assayed by a CO_2 fixation method using ^{14}C [187]. Changes in the activities of these enzymes were expressed as percentages to those of the LA-administered rats, when a 0.005 probability level was chosen.

VI-3 RESULTS

Distribution of radioactivity in hematic lipids of LAHPO-administered rats

LAHPO (170 kBq) was administered to 3 rats and blood was collected. The blood of rats contained 5.5 Bq/ml of radioactivity and 55% of the activity was moved to chloroform extract. The extract was subjected to the separation of lipid classes. Table VI-1 shows that the radioactivity was evenly dispersed in hematic lipids, phospholipids, galactolipids, triglycerides and diglycerides. Moreover, hydroxy

Table VI-1

Distribution of Radioactivity of Hematic Lipids of LAHPO-administered Rats

Lipid	Distribution %
Phospholipids	17.0
Galactolipids	15.7
Triglycerides	33.4
Diglycerides	11.4
Monoglycerides	0.9
Free FA	5.3
Hydroxy FA*	14.6
Steroid esters	1.8

*A band on TLC, whose Rf value was near that of free FA, was scraped off,extracted, methylesterified and analyzedwithGC-MS.

FA was detected and the radioactivity occupied 15%. Thus, it was considered that LAHPO was easily reduced to hydroxy FA and then metabolized to lipids.

Isolation of TBARS in blood of SP-administered rats

SP was administered to rats and 26 g and 717 Bq of blood was collected. The chloroform extract of blood contained 225 Bq of radioactivity and subjected to gel permeation chromatography (Fig. VI-1). The radioactive substances in

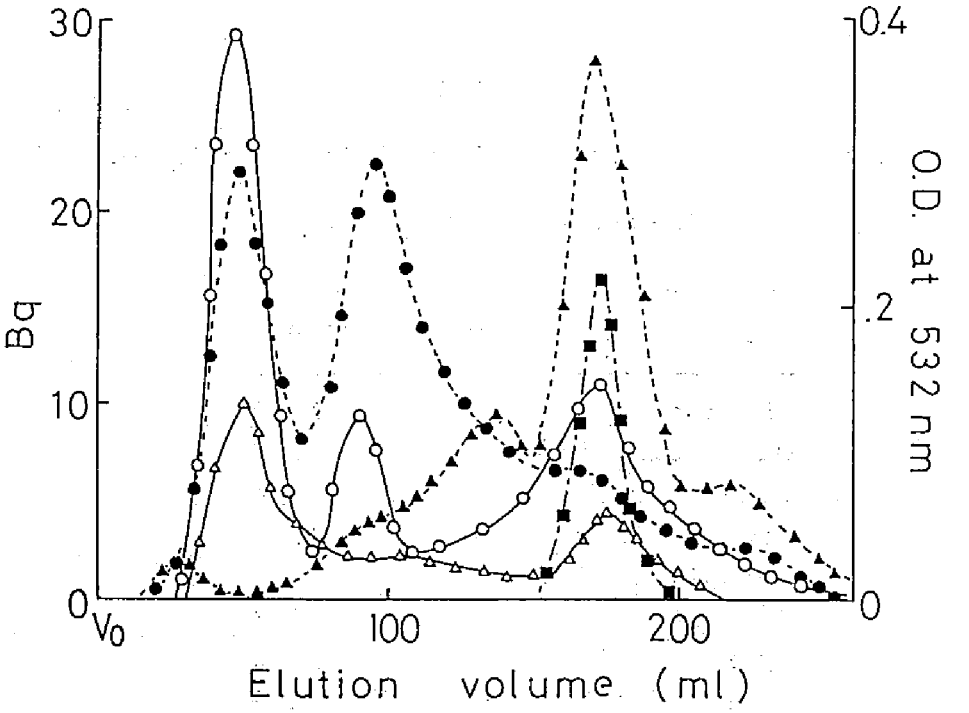


Fig. VI-1. Sephadex G-10 Chromatography of Blood. The chloroform extract of blood was permeated by the gel (detection by radioassay; —○— and by TBA test; —△—). A water extract of 50 mg of radioactive SP was also permeated (radioassay; ----●----, TBA test; ----▲----). Authentic MA was also applied (—·—■—·—).

blood showed three elution peaks at 30-70 ml, 80-100 ml and 160-190 ml. This pattern of radioactivity was similar to the pattern given by the water extract of SP. The third peak contained TBARS and its elution position agreed with low molecular-weight aldehyde such as MA. However, aldehyde was not identified by the GC-MS analysis. It was considered that various contaminants interfered the analysis because the amount of aldehyde might be very small.

Separation of SP-containing fraction from total lipids

The orally administered SP was accumulated in the hepatic mitochondria and microsomes after 12-24 h (Chapter V). Then, an attempt was made to separate the SP-containing fraction from lipids of these hepatic organelles. The SP-containing fraction was easily obtained using silica gel column chromatography (Table VI-2). SP was present in the acetone eluate and hydroperoxides free. Neutral lipids were eluted in the chloroform fraction and phospholipids were in the methanol fraction [175]. Their hydroperoxides were present mainly in fractions of the corresponding lipids. Galactolipids were eluted in the acetone fraction. The galactolipids occupied only 3% of the total lipids of hepatic organelles in rats. Then, TBARS amounts in these acetone fractions of organelles of SP-administered rats were measured

Table VI-2

Chromatographic Separation of SP from Total Lipids

Sample Applied amount	SP kBq	LAHPO	TL-HPO*	PE-HPO*
		PV (meq)		
	11.7	870	399	190
Elute with				
Chloroform	0.1	797	308	12
Acetone	11.4	39	59	19
Methanol	0.1	16	16	140

*Abbreviation of hydroperoxide.

**The added BHT was eluted in chloroform fraction.

Table VI-3

Specific Activity of TBARS in Acetone Fractions

	Mean±SE Bq/neq of MA (n=4)
Mitochondria	45.4±6.9
Microsomes	47.5±6.1
Authentic SP	47.9±0.9

(Table VI-3). Specific activity of TBARS in mitochondrial acetone eluate was 45.4 Bq/neq and closed to the value of SP per se (47.9 Bq/neq). That of microsomal TBARS was 47.5 Bq/neq and the same as SP per se.

Detection of hexanal and 9-ONA in hepatic organelles

These acetone fractions were submitted to the GC-MS analyses. The acetone fractions were methylesterified by diazomethane gas to avoid interference by galactolipids. In the acetone fractions of 3 h-microsomes, 7 h-mitochondria and 7 h-microsomes, the trace of a peak agreeing with authentic hexanal was observed on GC (Table VI-4). This peak was

Table VI-4

Detections of Hexanal and 9-ONA from Acetone Fractions of Mitochondrial and Microsomal Lipids

Detection of:	Hexanal	9-ONA	Hexanal	9-ONA
from:	Mitochondria		Microsomes	
after (h)				
3	-	-	+	-
7	+	-	+	-
12	+++	++	++	-
24	-	-	-	-

-; Not detected. +; Some fragment ions were detected in 10 Bq of samples. ++, +++; Identified in 20 Bq of samples.

introduced to MS and some ion, m/z: 43, 58 and 71, were detected. These ion peaks were the major fragment ions of hexanal [80]. In the acetone fraction of 12 h-mitochondria, two GC peaks were observed. Their relative retention times on GC and their fragmentation on MS completely agreed with those of authentic hexanal and 9-ONA methyl ester [80]. In the 12 h-microsomes, one peak was observed and identified as hexanal. From the acetone fractions of 24 h-organelles, no aldehydes were detected.

Changes in hepatic enzyme activity

Hepatic enzyme activities were measured in rats after 12-15 h of the administration with 350-500 mg each of SP. Mainly 13 kinds of enzymes indicated remarkable changes in the activity (Table VI-5). The activities of two cytosolic enzymes were stimulated. Glutathione peroxidase increased by 280% and catalase increased by 44%. The other detoxificases, superoxide dismutase and aldehyde dehydrogenase decreased by 40 and 22%. Glycolysis and pentose phosphate cycle enzymes also decreased by about 20%. The enzyme activities of mitochondrial tricarboxylic acid cycle, isocitrate dehydrogenase was activated and succinate dehydrogenase was inactivated. Mitochondrial aldehyde dehydrogenase was markedly inactivated. Both lipolysis and

Table VI-5

Changes in Activity of Hepatic Enzyme

Enzyme	Changes in activity (%)*
Cytosolic	
Glutathione peroxidase	280
Catalase	144
Superoxide dismutase	60
Aldehyde dehydrogenase (NADP-dependent)	78
Phosphofructokinase	72
Hexokinase	75
Glucose-6-phosphate dehydrogenase	85
Mitochondrial	
Isocitrate dehydrogenase	135
Succinate dehydrogenase	62
Cytochrome C oxidase	136
Aldehyde dehydrogenase (NAD-dependent)	55
Carnitine palmitoyl CoA transferase	138
Microsomal	
Acetyl CoA carboxylase	165

*Change % to the activity (100%) of LA-administered rats (n=8) when a 0.005 probability level was chosen.

lipogenesis enzymes were remarkably stimulated. The activity of mitochondrial carnitine palmitoyl CoA transferase increased by 38% and microsomal acetyl CoA carboxylase increased by 65%. Thus, while aldehyde dehydrogenases suffered injury by the intake of aldehyde-rich SP, the lipid metabolism enzymes were stimulated.

Incorporation of radioactivity into lipids

SP was administered to rats and the distribution of radioactivity was observed in the mitochondrial and microsomal lipids after 12 h (Table VI-6). Fifty percents in the radioactivity were incorporated into neutral lipids both of mitochondria and microsomes. The radioactivity in the acetone fraction seemed to be attributed to SP. Twenty

Table VI-6

Distribution of Radioactivity in Hepatic Mitochondria and Microsomal Lipids of SP-Administered Rats

	Distribution (%)	
	In Mitochondria	Microsomes
Triglycerides	37	41
Free FA	22	18
Steroid esters	6	7
Di and Monoglycerides	7	5
SP and Galactolipids	10	17
Phospholipids	9	23

percents of the activity were present in the microsomal phospholipids.

Time courses of the changes in radioactivity containing in these organelle lipids were observed (Fig. VI-2). The incorporation of radioactivity into mitochondrial and microsomal lipids increased suddenly after 12 h of the

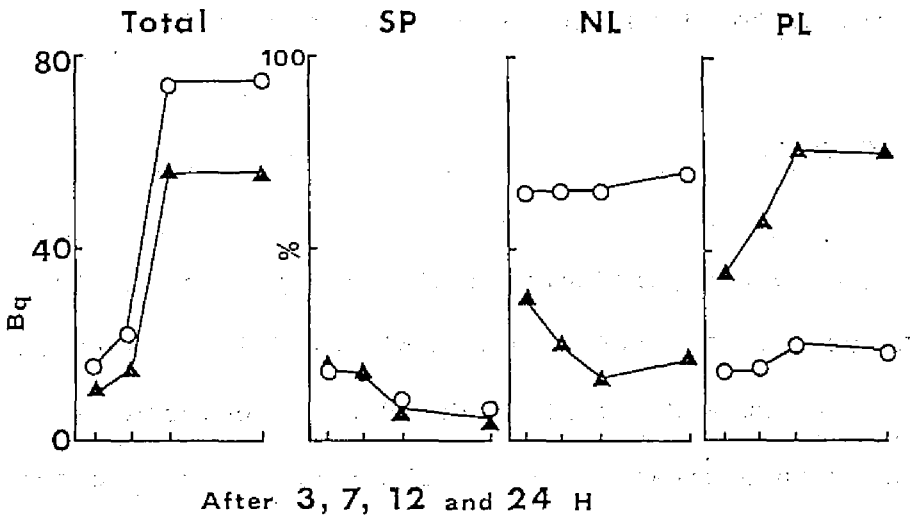


Fig. VI-2. Changes in Incorporated Radioactivity into Hepatic Mitochondrial and Microsomal Lipids in SP-Administered Rats. The total lipids were obtained by the chloroform extraction from hepatic mitochondria (○)and microsomes (▲) and applied on silica gel column chromatography. The chloroform, acetone and methanol eluates from the column were referred to fractions of neutral lipids (NL), SP and phospholipids (PL), respectively. The incorporation of radioactivity into these fractions were expressed as percentages occupied in the total lipids.

administration. The radioactivity in acetone eluate containing SP decreased from 20% (after 3 h of the administration) to 5% (after 24 h). The incorporation of radioactivity into neutral lipids indicated a high ratio in mitochondria after 3 h and the ratio was constant, while the incorporation ratio in microsomes decreased with time. The incorporation ratio into phospholipids was low in mitochondria and remarkably increased with time in

microsomes. Thus, it was considered that SP was detoxified and metabolized to lipids in the hepatic organelles.

VI-4 DISCUSSION

The autoxidation products of LA were separated into two parts, LAHPO and SP, and were administered intragastrically to rats. LAHPO was readily reduced or decomposed in the animal gastrointestinal tract. The reduced or decomposed products were rapidly metabolized to lipids (Table VI-1). On the contrary, SP, whose form was partly unchanged, seemed to be incorporated into blood (Fig. VI-1).

Then, an attempt to detect hexanal and 9-ONA which were the major components of SP (Chapter III) was made in liver. SP-containing fraction could be chromatographycally separated from lipids of the hepatic mitochondria and microsomes (Table VI-2). Specific activities of TBARS in the SP-containing fractions well agreed with that of SP per se (Table VI-3). Then, the SP-containing fraction was analyzed with GC-MS. Trace amount of hexanal or 9-ONA was identified (Table VI-4).

Peroxides such as LAHPO were present in living cells [188, 189]. LAHPO is decomposed and the main products were hexanal [24] and 9-ONA [42]. However, almost all of the peroxides was removed from the SP-containing fractions by

silica gel column chromatography of Table VI-2. Furthermore, BHT was added as antioxidant to these analytical systems. Therefore, hexanal and 9-ONA detected in Table VI-4 were originated from SP administered orally and never be artifacts during these manipulations. Draper and co-workers also indicated the incorporation of MA into cells [190-192]. Thus, the low molecular-weight aldehydes in SP were incorporated into rat liver.

The incorporated aldehydes stimulated the activities of lipogenic and lipolytic enzymes (Table VI-5). The stimulation of these enzymes were always observed by the administrations of autoxidized oils [193-197]. The stimulation is considered to be due to repair the biomembrane. The radioactivity of SP was incorporated into microsomal phospholipids (Fig. VI-2). Therefore, the radioactive substances of incorporated SP may be utilized for the membrane repair.

The incorporation of radioactivity into liver reached a maximum after 12-24 h (Chapter V). Hexanal and 9-ONA were detected in 12 h-mitochondria and -microsomes, but not 24 h-organelles. The radioactivity was dispersed in lipids after 14 h of SP administration (Table VI-6). The radioactivity of the acetone fraction decreased with time proceedings (Fig. VI-2) and the aldehyde dehydrogenases were remarkably inactivated (Table VI-5). Therefore, aldehydes such as

hexanal and 9-ONA seemed to be detoxified and metabolized in the hepatic organelles.

It was concluded that the orally administered SP was detoxified in hepatic organelles and then gave a damage to biomembranes. The membranes were repaired and subsequently, lipogenic and lipolytic enzymes were stimulated. Furthermore, these toxic components to the biomembrane should be investigated [198, 199]. One of the toxicants might be 9-ONA as reported by author group [200].

VII SUMMARY

Oral toxicity of the autoxidation products of linoleic acid (LA) to rats was investigated. LA reacted easily with one mole of atmospheric oxygen and formed linoleic acid hydroperoxide (LAHPO). LAHPO further reacted with oxygen and polymerized or decomposed. The reaction of LAHPO with oxygen followed the first-order with respect to both LAHPO and oxygen and was a bimolecular reaction. The various kinds of compounds produced by the latter reaction are generally termed secondary autoxidation products (SP). SP was a complex mixture and aldehyde-rich. SP consisted of 38.2% polymers, 26.5% epoxyperoxides or endoperoxides, 6.2% short chain (C_6-C_{10}) carboxylic acids, 5.0% 9-oxononanoic acid (9-ONA), 3.8% hexanal, 2.6% nonanedioic acid, 0.9% 8-oxooctanoic acid, 0.3% 12-oxododecadienoic acid and so forth.

These autoxidation products were reactive to proteins. Lysozyme was inactivated and its basic amino acid residues were damaged by these products in the order of SP > LAHPO > LA. The damage seemed to be caused by Schiff base formation of basic amino acids with aldehydes. Casein easily incorporated these autoxidation products in the above order and its conformation was changed. These autoxidation products were orally administered to rats. SP was most

toxic to the rat growth. A triple dose of SP killed the animals. It seemed that death resulted when the aldehydes damaged the protein of gastrointestinal mucosa.

However, a single dose of 100 mg each of the autoxidation products had no effect on the rat growth. Then, this amount each of [U-^{14}C]LAHPO and SP were administered intragastrically and their incorporation into body was radiochemically compared with that of LA. Almost all of the radioactive substances of LAHPO was incorporated and excreted through urine and respiration as well as in the case of LA. LAHPO, however, seemed to be easily reduced or decomposed in the animal gastrointestinal tract. On the contrary, half of the radioactive substances of SP was incorporated, of which 1/2 was excreted through urine and 1/4 through CO_2. After 12-24 h, 2.6% of the administered SP had accumulated in liver and the major components of SP, hexanal and 9-ONA, were found in the hepatic organelles. It was concluded that the low molecular-weight components of SP, whose forms were partly unchanged, were absorbed into blood and further incorporated into hepatic mitochondria and microsomes.

Enzyme activity of the mitochondrial aldehyde dehydrogenase decreased with the SP dose. The incorporation of radioactivity of SP into the microsomal phospholipids was observed. Thus, the aldehydes composed in SP seemed to

be incorporated, detoxified in the hepatic mitochondria and metabolized to lipids in the microsomes.

The SP dose impaired hepatic functions. The amount of thiobarbituric acid reactive substances remained high even after the disappearance of the radioactivity. An elevation of serum transaminase activities also continued. A hypertrophy of liver (1.5-fold) was observed after 72 h but recovery occurred after 96 h. Furthermore, the activities of carnitine palmitoyl CoA transferase and acetyl CoA carboxylase were noticeably stimulated. Therefore, it was concluded that although the orally administered SP damaged biomembrane of the hepatic organelles, the biomembrane was repaired.

On the basis of these data, the fate of orally administered SP in rat liver is summarized as follows: 1. SP was more reactive to proteins than LAHPO. 2. The low molecular-weight aldehydes in SP accumulated in the hepatic organelles after 12-24 h. 3. The liver underwent damage by these aldehydes after 24-48 h and the damage was caused by impairment of biomembrane. 4. The lipogenic enzymes were remarkably stimulated to repair biomembrane. and 5. After 96 h of the administration, the hepatic function was recovered.

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